Stability and antiviral activity against human cytomegalovirus of artemisinin derivatives

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Objectives: Artesunate, a derivative of dihydroartemisinin, itself a product of artemisinin, inhibits the replication of cytomegalovirus in vitro. In vivo, artesunate undergoes rapid conversion into the active metabolite dihydroartemisinin. The in vitro stability of the compounds and the antiviral activity of dihydroartemisinin are of great concern for the interpretation of in vitro testing. The aim of the study was to measure artesunate conversion into dihydroartemisinin in culture medium and to evaluate the stability and antiviral activity of artemisinin derivatives, according to culture conditions.

Methods: Conversion of artesunate into dihydroartemisinin was measured in culture medium with or without fetal calf serum, in the presence or absence of fibroblast monolayers, at different times. The stability of artemisinin derivatives was determined in serum-enriched medium. Concentrations of each compound inhibiting viral DNA synthesis by 50% were determined in fibroblasts cultured in serum-free or serum-enriched medium, after addition of compound as a single dose or fractional doses.

Results: Conversion of artesunate into dihydroartemisinin in serum-free or serum-enriched medium was non-equimolar. The half-lives of artesunate, dihydroartemisinin and artemisinin were 10.3 ± 0.9, 5.2 ± 0.5 and 11.2 ± 1.2 h, respectively. Activity of dihydroartemisinin and artesunate was markedly reduced in serum-starved cells. Unexpectedly, dihydroartemisinin displayed a lower activity than artesunate. Addition of both compounds as fractional doses increased their activity. Artemisinin had no anticytomegaloviral activity.

Conclusions: Artemisinin derivatives were shown to be unstable in vitro and their addition as fractional doses could partly compensate for this instability. Importantly, the cellular physiological condition was a determinant of their antiviral activity.

Keywords: antiviral therapy, metabolism, HPLC, drug susceptibility

Introduction

Human cytomegalovirus (HCMV) is a widespread opportunistic pathogen in immunocompromised individuals, including allograft recipients and patients with AIDS. It remains the leading cause of congenital viral infections and may be responsible for severe disease leading to death or serious neurodevelopment sequelae in infants infected in utero. Antiviral drugs currently available in clinical practice include ganciclovir (and its prodrug valganciclovir), foscarnet and cidofovir, which all target the viral DNA polymerase. Their clinical use is limited by severe adverse side effects and the possible emergence of resistance.1 Artesunate, an antimalarial drug, has been shown to inhibit the replication of both HCMV reference strains and clinical isolates susceptible or resistant to ganciclovir in fibroblasts and U373MG astrocytoma cells.2-7 The intensive use of artesunate in patients with malaria has demonstrated its high safety and tolerability profile.8,9 Thus, it has been proposed as an alternative to the currently available antiviral drugs in
patients failing therapy.\textsuperscript{1,10,11} Artesunate can be administered orally, intramuscularly, intravenously and by the rectal route.\textsuperscript{12,13}

Artesunate is a hemisuccinate derivative of dihydroartemisinin, itself obtained by the reduction of artemisinin, a natural sesquiterpene lactone extracted from \textit{Artemisia annua}, a Chinese herbal medicine used as a traditional antimalarial.\textsuperscript{14–16} The pharmacophore of artemisinin derivatives is a 1,2,4-trioxane ring incorporating an endoperoxide bridge essential for antimalarial activity.\textsuperscript{17,18} Within the infected erythrocyte, the reduction of the peroxide bridge, favoured by ferrous iron (Fe\textsuperscript{2+}), and the generation of carbocentered radical molecules and reactive oxygen intermediates are thought to be crucial to the antimalarial activity.\textsuperscript{17,19} In addition to their antimalarial activity, artemisinin and its derivatives also have anticancer properties.\textsuperscript{13,20–23}

The \textit{in vitro} antiviral activity of artemisinin derivatives was described in 1982.\textsuperscript{24} Their mechanism of action on the replication of HCMV is incompletely known. Artesunate inhibits the \textit{in vitro} replication of HCMV at an early stage, by mechanisms linked to cell activation pathways.\textsuperscript{4,5} The products of the major immediate early (MIE) IE2 and IE1 genes, which are multifunctional proteins involved in regulating both viral and cellular gene expression, are essential for the progression of the replication cycle.\textsuperscript{25} Their transcription is driven by the complex and potent MIE promoter (MIEP), which contains binding sites for a diverse set of signal-regulated stimulatory and inhibitory transcription factors. Among these, cellular factors NF-κB and Sp1, which are overexpressed through the initial binding of viral envelope glycoproteins to cellular receptors, activate the MIEP. Artesunate was shown to markedly reduce both the protein level and the binding activity of these virus-induced cellular factors and to inhibit IE2 expression.\textsuperscript{26} However, He et al.\textsuperscript{26} did not find a significant effect of artesunate on MIE gene transcripts. The peroxide bridge is thought to play a role in anti-HCMV activity, as (i) suppression of HCMV replication is enhanced by the addition of Fe\textsuperscript{2+},\textsuperscript{24,5,26} (ii) dimers derived from artemisinin with two endoperoxide bridges have been reported to be more effective at inhibiting HCMV replication than their monomeric counterparts\textsuperscript{2} and (iii) a deoxy dimer lacking the endoperoxide bridge did not have any anti-HCMV activity.\textsuperscript{27}

In vivo, artesunate is rapidly and almost completely converted into dihydroartemisinin, mainly by plasmatic and cellular esterases.\textsuperscript{28–30} After artesunate intravenous injection, plasmatic artesunate and dihydroartemisinin half-lives are <15 and 60 min, respectively.\textsuperscript{11,12,28,30} Thus, artesunate acts as an ester prodrug and its main metabolite, dihydroartemisinin, is considered the active compound \textit{in vivo}. Unexpectedly, the \textit{in vitro} anti-HCMV activity of dihydroartemisinin was shown to be much lower than that of artesunate\textsuperscript{3} and in spite of a chemical structure close to that of artemisinin, inconsistent results concerning artemisinin anticytomegaloviral activity have been reported by Arav-Boger et al.\textsuperscript{3} and Chou et al.\textsuperscript{3} The basis for all these discrepancies is unknown. Artemisinin derivatives are known to be unstable compounds, especially dihydroartemisinin.\textsuperscript{14} \textit{Ex vivo}, artesunate can also form dihydroartemisinin through chemical hydrolysis and plasma esterase-mediated hydrolysis. In culture medium, the stability of the artemisinin derivatives as well as the level of artesunate conversion into dihydroartemisinin are unknown, however, they are of major concern for the interpretation of \textit{in vitro} antiviral activity testing. The aims of the study were to measure artesunate conversion into dihydroartemisinin in culture medium as well as hydrolysis catalysed by fetal calf serum (FCS) esterases and to evaluate the stability and antiviral activity of artemisinin derivatives in different experimental conditions.

### Materials and methods

#### Cells and viruses

Human lung fibroblasts (MRC-5; FreshFrozenCells\textsuperscript{8}, Diagnostics Hybrids) — purchased from bioMérieux-Argène (Verniolles, France) — were grown in minimum essential medium (Invitrogen, Saint-Aubin, France) containing variable amounts of inactivated FCS (Pan Biotech, distributed by Dutscher, Brumath, France). FCS inactivation was achieved by heating at 56 °C for 30 min.

Reference HCMV strain AD169 (ATCC VR-538; Rockville, MD, USA) and a clinical isolate (VI1210) susceptible to antiviral drugs were propagated in MRC-5 cells.

#### Compounds

Ganciclovir, artesunate, dihydroartemisinin and artemisinin were purchased from Sigma (Saint-Quentin Fallavier, France). Ganciclovir was prepared as a 10 mM aqueous solution. Artesunate, dihydroartemisinin and artemisinin were dissolved in DMSO to constitute 100 mM solutions. All compound stocks were stored at −80°C.

#### Antiviral assay

Antiviral activity was assessed by measuring viral DNA synthesis in the presence and the absence of the drug after 4 days of incubation. The drug concentration inhibiting viral DNA synthesis by 50% (IC\textsubscript{50}) was determined by a previously described real-time PCR-based assay.\textsuperscript{1} Briefly, MRC-5 fibroblasts grown as monolayers in 24-well plates were inoculated with cell-associated virus (5000 cells per well) or cell-free virus (multiplicity of infection 0.1). After virus adsorption, cells were fed with medium containing serial 2-fold dilutions of each test compound. Each drug concentration was tested in quadruplicate.

To test the influence of the FCS concentration upon drug antiviral activity, experiments were carried out in parallel with culture medium containing no serum, 2% FCS or 10% FCS (inactivated or not).

To test the influence of the mode of drug addition on the cell cultures, experiments were conducted in parallel to compare single dosing (as described above) with fractional dosing. Fractional dosing was performed as follows: after virus adsorption, cells were fed with medium containing one-quarter of the final dose of the test drug and three additional fractional doses were consecutively added to the cell culture medium at 24, 48 and 72 h post-infection in a 10 μl volume for a total medium volume of 1 ml per well.

After 4 days of incubation, the cells were lysed. The number of HCMV genomes was quantified in cellular lysates by real-time PCR. Determination of the IC\textsubscript{50} values and statistical analysis were carried out using the computer package Ph-fit.\textsuperscript{32} Growth inhibition (I) and the corresponding drug concentrations (C) were fitted according to a sigmoid model, described as: \(I = \frac{(I_{\text{max}} - C) / IC_{50}}{C + IC_{50}^2}\), where \(I_{\text{max}}\) is the maximum growth inhibition and \(y\) is the sigmoid factor of the curve.

#### Cytotoxicity assay

The cellular cytotoxicity of the drugs was measured using the CytoTox96\textsuperscript{9} Non-Radioactive Cytotoxicity Assay (Promega, Charbonnieres, France) as reported by Effert et al.\textsuperscript{4} The assay determines lactate dehydrogenase (LDH) activity in the residual cells after incubation with increasing concentrations of the test compound. Briefly, fibroblasts were grown in 24-well plates to confluence as for antiviral assays and then incubated at 37 °C for 4 days in culture medium containing serial dilutions of artesunate,
Ganciclovir was tested in parallel as a control.

ART, artesunate; DHA, dihydroartemisinin; GCV, ganciclovir; ND, not determined.

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**Effect of FCS concentration on cellular metabolism**

To evaluate the effect of the serum concentration on cell metabolism, LDH activity was measured as indicated above in mock-infected cells cultured for 4 days in drug-free medium containing 0%, 2% or 10% inactivated FCS. LDH activity was also measured in cell culture supernatants by using the CytoTox96® Non-Radioactive Cytotoxicity Assay. Supernatants (50 μL per well of a 24-well plate) were transferred to the wells of a 96-well flat-bottomed plate and mixed with an equal volume of substrate. The mixtures were incubated for 30 min at room temperature in the dark. Then, 50 μL of stop solution was added to each well and the colour reaction was quantified using an ELISA reader (optical density (OD) at 490 nm).

**Effect of FCS concentration on viral genome synthesis**

Fibroblast monolayers grown to confluence in 24-well plates were inoculated with strain AD169 and then incubated in parallel in the presence of 0%, 2% or 10% inactivated FCS, or in the absence of FCS. After 4 days of incubation in the three culture conditions tested, cells were lysed and the number of HCMV genomes was quantified in cellular lysates by real-time PCR. The means (±SD) of the ODs obtained in three independent experiments were calculated.

**Determination of artesunate and dihydroartemisinin in culture medium**

Dilutions of artesunate (1.88 and 30 μM) were prepared in culture medium containing 0% or 10% (inactivated or not) FCS. For each condition, a 600 μL aliquot was immediately stored at −80°C. The aliquots were withdrawn after 30 min of incubation and then every hour until 8 h and every day until 4 days. The aliquots were immediately stored at −80°C.

Concentrations of artesunate and dihydroartemisinin in culture medium aliquots were measured by HPLC with electrochemical detection. The procedure involved solid-phase extraction on an Oasis® HLB 1 cc/30 mg cartridge (Waters, Milford, MA, USA) and artemisinin was used as the internal standard. Independent of the concentrations, the extraction ratios were >85% for the three analytes. Chromatographic separation of the analytes was achieved on an analytical 150×4.6 mm i.d. Thermo BDS Hypersil Cyano column, 5 μm particle size (Thermo Fischer Scientific Inc., Waltham, MA, USA) with a mobile phase of acetonitrile/0.01 M sodium acetate buffer at pH 4.65 (30:70, v/v) and at a flow rate of 1 mL/min. The column eluate was monitored in reductive mode at −800 mV (Decade II, Antec Leyden, The Netherlands). Linearity over the concentration ranges of 5–500 and 10–500 ng/mL was obtained for dihydroartemisinin and artemisinin, respectively. The back-calculated concentrations of the calibration standards were within ±10% of the nominal value with a coefficient of variation value of <10%. Data were acquired and analysed using Chromeleon® 6.80 software (Dionex Corporation, Sunnyvale, CA, USA). The impact of the medium composition (i.e. presence of FCS and/or cells) on the stability of artesunate was compared by using area under the concentration versus time curves (AUCs) from 0 to time t. AUC_{0–t} was determined according to the trapezoidal rule. The degradation rate (k) was obtained as the slope of the linear regression of the log-transformed concentration versus time data in the terminal portion of the curve. The half-life (t_{1/2}) was estimated from the k constant using t_{1/2} = 0.693/k. One-way ANOVA and a simple contrast test were used to compare experimental conditions. A P value <0.05 was considered as statistically significant.

In addition, to assess the stability of artesunate derivatives, media containing artesunate, dihydroartemisinin and artemisinin (1.88 μM) and 10% inactivated FCS were incubated at 37°C without fibroblasts. Drug concentrations were measured at different incubation times.

**Results**

**Antiviral activity of artesunate, dihydroartemisinin and artemisinin according to FCS concentrations**

The results are presented in Table 1. AD169 replication suppression by artesunate was significantly reduced in the absence of FCS.

**Table 1. Activity of artesunate derivatives against HCMV reference strain AD169 and clinical isolate VI1210 according to the percentage of inactivated FCS in the cell culture medium**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Drug</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comparison between 0%, 2% and 10% FCS&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0% FCS</td>
<td>2% FCS</td>
</tr>
<tr>
<td>AD169</td>
<td>ART</td>
<td>22.19 ± 9.89</td>
<td>5.57 ± 2.91</td>
</tr>
<tr>
<td></td>
<td>DHA</td>
<td>47.06 ± 13.79</td>
<td>22.56 ± 7.22</td>
</tr>
<tr>
<td></td>
<td>GCV</td>
<td>0.65 ± 0.24</td>
<td>0.37 ± 0.21</td>
</tr>
<tr>
<td>VI1210</td>
<td>ART</td>
<td>9.50 ± 7.73</td>
<td>5.17 ± 2.29</td>
</tr>
<tr>
<td></td>
<td>DHA</td>
<td>10.62 ± 7.74</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>GCV</td>
<td>1.37 ± 0.41</td>
<td>ND</td>
</tr>
</tbody>
</table>

ART, artesunate; DHA, dihydroartemisinin; GCV, ganciclovir; ND, not determined.

Ganciclovir was tested in parallel as a control.

<sup>a</sup>Means ± SD from at least four independent experiments.

<sup>b</sup>A one-way ANOVA was used to compare the three culture conditions (0%, 2% and 10% FCS) and a Mann–Whitney test to compare culture conditions two by two. Significant P values (<0.05) are indicated in bold.
as compared with suppression in the presence of 2% or 10% inactivated FCS. For isolate V11210, artesunate IC$_{50}$ values were significantly lower in the presence of 10% FCS versus 0%. For both strains, dihydroartemisinin activity was also markedly reduced in the absence of FCS, but the difference was significant only for the AD169 strain. Artesunate and dihydroartemisinin were more effective at inhibiting the replication of the clinical isolate V11210 than the AD169 strain (in medium with 10% FCS, $P=0.03$ and $P=0.001$, respectively). Artemisinin had no antiviral activity and this result was confirmed by testing artemisinin purchased from another source (Sao Kim Pharma Co., Vinh Phuc, Vietnam) (data not shown). For ganciclovir, tested as control, IC$_{50}$ values for AD169 and V11210 were similar whatever the concentration of FCS in the culture medium. Serum inactivation had no effect upon the antiviral activity of artesunate, dihydroartemisinin and ganciclovir, as the respective IC$_{50}$ values of these compounds were similar whether FCS was inactivated or not (data not shown).

To check whether the reduction of antiviral activity in the absence of serum was due to the cytotoxicity of artesunate or dihydroartemisinin, an LDH-based cytotoxicity assay was applied. LDH activity was measured in serum-starved cells in the presence and the absence of the compounds. No reduction in LDH activity was observed in the presence of the compounds in the relevant range of concentrations between 1.88 and 60 $\mu$M when compared with the LDH activity in control cells not treated with the drugs (data not shown).

**Effect of FCS concentration on cellular metabolism**

LDH activity was significantly reduced ($P=0.001$) in serum-starved mock-infected cells (OD = 633.33 ± 102.64) as compared with LDH activity in cells cultured in the presence of 2% FCS (OD = 792.50 ± 79.49) or 10% FCS (OD = 1431.17 ± 62.36). LDH activity was unchanged in supernatants, whatever the FCS concentration in the culture medium (data not shown).

**Effect of FCS concentration on viral genome synthesis**

After 4 days of incubation of the infected cells in serum-free or serum-enriched medium (2% or 10%), genome synthesis was measured in cellular lysates in six independent experiments. The mean number of HCMV genomes was 10.15 ± 0.56 log$_{10}$ copies/mL in the absence of FCS, and 9.99 ± 0.67 log$_{10}$ copies/mL and 9.67 ± 0.72 log$_{10}$ copies/mL in the presence of 2% and 10% FCS, respectively ($P=0.068$; data not shown).

**Stability of artemisinin derivatives in medium**

The degradation of artesunate followed first-order kinetics independently of the experimental conditions (concentration of FCS in culture medium, presence or absence of fibroblasts) and the initial concentration of the compound (1.88 or 30 $\mu$M). For an initial artesunate concentration equal to 30 $\mu$M in medium supplemented with 10% inactivated FCS, the AUC values were 662 ± 15 and 532 ± 18 h·$\mu$M without and with fibroblasts, respectively (Figure 1). At 1.88 $\mu$M, these values were 44.3 ± 2.03 and 33.4 ± 2.1 h·$\mu$M, respectively. Thus, the AUC values were 27.6% ± 4.0% higher in the absence of cells than in their presence. The corresponding half-life values were 14.4 ± 1.8 versus 12.4 ± 1.6 h ($P=0.019$) in the absence and in the presence of cells, respectively (Figure 1). No statistically significant difference was observed between medium enriched or not with 10% FCS, inactivated or not (data not shown). Whatever the experimental conditions, the elimination kinetics was slightly faster in the presence of cells ($P<0.05$).

Dihydroartemisinin concentrations were low, whatever the initial artesunate concentration and the medium composition. The concentration versus time curves of dihydroartemisinin, expressed as percentages of the initial artesunate concentration, were not influenced by any experimental condition (data not shown). For initial artesunate concentrations of both 30 and 1.88 $\mu$M, a steady-state with maximal dihydroartemisinin concentrations (representing 10.3% ± 0.5% of the initial artesunate concentration) was obtained in a range of 6–24 h (Figure 1).

To assess the stability of dihydroartemisinin (independently of artesunate) and artemisinin, an additional study was performed. The stability of artemisinin derivatives was evaluated in medium supplemented with 10% inactivated FCS at 37°C over a 48 h period. The half-life values were 10.3 ± 0.9, 5.2 ± 0.5 and 11.2 ± 1.2 h for artesunate, dihydroartemisinin and artemisinin, respectively.

**Effect of addition of artesunate and dihydroartemisinin as fractional doses on antiviral activity**

Antiviral assay was performed as described above, except that artesunate and dihydroartemisinin were not applied as a single dose but as fractional doses (one-quarter of the final dose) consecutively added post-infection for 4 days. The results are shown in Table 2. Comparison between the modes of drug addition was performed in experiments carried out in parallel to rule out variability due to cell passages, inoculum calibration or medium preparation. For AD169, the IC$_{50}$ values were significantly lower when...
The AD169 strain. The addition of FCS enhanced replication suppression. The absence of FCS in the culture medium alters cellular metabolism, as reflected by the reduction of LDH activity without enzyme release in the supernatant in FCS-starved cells compared with cells cultured in the presence of 10% FCS. Furthermore, HCMV DNA synthesis remained unchanged whether or not FCS was present in the culture medium. As the artesunate conversion rate was similar in the presence or the absence of serum, these results confirm that artesunate as well as dihydroartemisinin activity depends on the physiological condition of the infected cell. In contrast, the antiviral activity of ganciclovir, which specifically inhibits the viral DNA polymerase, remained similar in the absence of FCS. In vivo, HCMV infects a wide variety of cells. A major problem is therefore whether artesunate is able to inhibit HCMV replication in these target cells, whatever their physiological condition.

Dihydroartemisinin was less efficient than artesunate in suppressing AD169 replication, as shown by their respective IC50s. Dihydroartemisinin has been reported to be even less efficient by Chou et al., who observed 25% inhibition of AD169 replication at 15 μM. Methodological differences between both studies, such as the type of cells, culture time and mode of revelation, might have contributed to these differences. The lower antiviral activity of dihydroartemisinin was unexpected, since once inside the cells artesunate is hydrolysed to dihydroartemisinin and hemisuccinic acid by cellular esterases. However, dihydroartemisinin is more unstable than artesunate in culture medium. Moreover, cellular uptake of dihydroartemisinin is likely lower as its chemical structure lacks a hemisuccinate moiety. Altogether, these facts could explain the less efficient suppression of HCMV replication in vitro by dihydroartemisinin. Applying artesunate or dihydroartemisinin in fractional doses increased their antiviral activity. This finding is consistent with the report by Chou et al., who found artesunate to be more potent against AD169 when applied as fractional doses. However, the difference in the artesunate activity according to the mode of dosing was not significant for the clinical isolate studied, whereas the increase of dihydroartemisinin antiviral activity was marked for both strains. These facts could result from both the high susceptibility of VI1210 to artesunate and the great

**Table 2.** Effect on antiviral activity of addition of artesunate or dihydroartemisinin as fractional doses in the culture medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Drug</th>
<th>IC50 (μM)b</th>
<th>Comparison between the two culture conditions (P)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>single dose</td>
<td>fractional doses</td>
</tr>
<tr>
<td>AD169</td>
<td>ART</td>
<td>6.13 ± 0.87</td>
<td>4.01 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>DHA</td>
<td>20.17 ± 9.58</td>
<td>2.88 ± 1.88</td>
</tr>
<tr>
<td></td>
<td>GCV</td>
<td>1.48 ± 0.18</td>
<td>2.87 ± 1.10</td>
</tr>
<tr>
<td>VI1210</td>
<td>ART</td>
<td>1.94 ± 1.30</td>
<td>1.22 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>DHA</td>
<td>5.18 ± 2.89</td>
<td>1.49 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>GCV</td>
<td>1.24 ± 0.28</td>
<td>1.69 ± 0.45</td>
</tr>
</tbody>
</table>

ART, artesunate; DHA, dihydroartemisinin; GCV, ganciclovir. Ganciclovir was tested in parallel as a control.

*Means ± SD from four independent experiments.*

*Drug addition in the culture medium: as single dose or four fractional doses.*

*Significant P values (<0.05) are indicated in bold.*

Numerous studies have shown the ability of artesunate to inhibit HCMV replication in vitro. In vivo, dihydroartemisinin is the main artesunate metabolite. In vitro, only a partial artesunate conversion into dihydroartemisinin was observed in tissue culture medium whether FCS was present or not. With or without serum, the sum of the respective concentrations of artesunate and dihydroartemisinin decreased rapidly, suggesting degradation of the molecules due to their intrinsic chemical instability. Dihydroartemisinin is particularly unstable because of the lactole function present in its chemical structure. The transition from conformation α to conformation β (flip-flop) generates constraints on the endoperoxide bridge, which can then break. In the presence of cells, the artesunate concentration in the medium decreased significantly faster than in the absence of cells, whereas dihydroartemisinin concentration did not increase, suggesting weak intracellular uptake of artesunate. Unfortunately, methods to measure intracellular artesunate-derived concentrations are not yet available. To date, fibroblast uptake of artesunate has not been studied. An earlier study reported Caco-2 intestinal cell uptake of artesunate by passive diffusion. In the present study, the degradation kinetics of dihydroartemisinin and artesunate had similar profiles under all the conditions tested. A striking finding of the study is the influence of the amount of FCS in the tissue culture medium on the antiviral activity of artesunate and dihydroartemisinin. In the absence of FCS, artesunate was shown to inhibit the replication of both reference strain AD169 and a clinical isolate at concentrations higher than those inhibiting replication in the presence of 10% FCS. A similar result was found for dihydroartemisinin when testing the artesunate or dihydroartemisinin was added as fractional doses. For VI1210, the IC50 values of artesunate and dihydroartemisinin were also lower when the drug was added as fractional doses; however, the difference was significant only for dihydroartemisinin. Considering ganciclovir, IC50 values were higher when the drug was added as fractional doses. However, the difference attained significance only for strain AD169 (P = 0.046).

**Discussion**

The high susceptibility of VI1210 to artesunate and the great...
instability of dihydroartemisinin. In contrast, ganciclovir was less efficient in suppressing HCMV replication when applied as fractional doses. These findings suggest that fractional dosing could partly compensate for the instability of the molecules, in particular that of dihydroartemisinin.

The chemical structures of artesunate, dihydroartemisinin and artemisinin are very similar and the three molecules contain the same endoperoxide bridge. However, artemisinin had no antiviral activity. Our results are in agreement with those of Chou et al. However, they are inconsistent with those reported by Arav-Boger et al., who showed similar IC50 values for artesunate and artemisinin. It is unclear why artemisinin does not inhibit the replication of HCMV with the same efficiency as artesunate in the present study. Both molecules had similar stability in the culture medium. Neither induced cytotoxicity in the relevant range of concentrations tested for phenotyping. In the cell culture conditions, very poor cell uptake induced cytotoxicity in the relevant range of concentrations tested for phenotyping. In the cell culture conditions, very poor cell uptake of artesunate might be implied.

Seven immunocompromised patients have received artesunate to treat HCMV infection. In two of them, viral replication was controlled, whereas in the other five, treatment was ineffective. Six of them received oral artesunate and one intravenous artesunate. Regarding the in vivo short half-lives of artesunate (15 min) and dihydroartemisinin (60 min) and our experimental data, which are in favour of a time-dependent effect of artemisinin derivatives on HCMV, artesunate oral administration twice or thrice daily could be more effective than artesunate administration once daily. Although artesunate has been shown to be well tolerated when administered for the treatment of malaria, the safety of a new administration schedule should be assessed, in particular for long-term therapies. Thus, artesunate, a molecule with anti-HCMV activity, could contribute to a better management of HCMV-related diseases in immunocompromised patients.

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Transparency declarations
None to declare.

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