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The anti-malaria drug artesunate inhibits replication of cytomegalovirus in vitro and in vivo

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Abstract

Treatment of human cytomegalovirus (HCMV) infections with any of the currently available antiviral agents is frequently associated with the occurrence of severe complications, seriously threatening the successful outcome of treatment. Therefore, the development of novel antiviral strategies is a challenging goal of current investigations. Previously, we reported that artesunate (ART) is an effective, non-cytotoxic inhibitor of HCMV in vitro. Here, we demonstrate that the efficacy of the antiviral effect of ART is augmented by co-treatment of HCMV-infected fibroblasts with ferrous iron, i.e. Ferrosanol™, and/or the iron transfer-mediating molecule holo-transferrin. This could alleviate the HCMV-induced modulation of cell surface expression of adhesion molecule Thy-1, suggesting that ART might be able to prevent pro-inflammatory effects of infection. The iron-enhanced, antiviral effect of ART could also be demonstrated in cultured cells infected with rat cytomegalovirus. Experiments using the RCMV/rat model showed that both the viral DNA load and virus titers in the salivary glands from infected rats were significantly reduced upon treatment with ART. Furthermore, an additive antiviral effect for ART together with each one of conventional anti-HCMV drugs, i.e. ganciclovir, cidofovir or foscarnet, was detected in HCMV-infected fibroblasts. These findings might open new perspectives regarding the use of ART in clinical trials.

Keywords: Cytomegalovirus; Drug development; Artesunate; Ferrous iron; Combined antiviral therapy

1. Introduction

Human cytomegalovirus (HCMV) is a herpesvirus that causes life-long infections in approximately 50% (40–80%) of the populations in Europe and North America (Onorato et al., 1985). In most cases, HCMV infection is asymptomatic, however, in individuals with an immature or compromised immune system such as neonates, transplant recipients and AIDS patients, a serious or even life-threatening disease may develop. Currently, a limited number of drugs is used for the treatment of a systemic or locally reactivated HCMV infection, i.e. (i) ganciclovir (GCV), (ii) its prodrug valganciclovir, (iii) cidovovir (CDV), (iv) foscarnet (FOS) and (v) fomivirsen. Nevertheless, each of these drugs is associated with a number of disadvantages. First, these antiviral compounds are usually administered intravenously or intravitreally, except for valganciclovir, which possesses improved oral bioavailability. In addition, prolonged treatment with each one of these drugs is frequently followed by serious side effects. Moreover, GCV, valganciclovir, CDV and FOS have similar mechanisms of action by targeting, either directly or indirectly, the viral DNA polymerase. Treatment with any of these antiviral agents may therefore ultimately result in the emergence of single- or multi-resistant HCMV mutants. These considerations have promoted an intense search for novel therapeutic agents that are safe, potent and act at alternative antiviral targets.

Previously, we found that artesunate (ART), which is commonly used in the treatment of severe malaria, displays

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antiviral activity against HCMV in vitro (Efferth et al., 2002). The primary determinant of the cytotoxic activity of ART against malaria is ferrous iron (Fe^{2+}) (Cumming et al., 1997; van Agtmael et al., 1999). Fe^{2+} is released from infected erythrocytes upon *Plasmodium*-mediated hydrolysis of haemoglobin and catalyzes the cleavage of the intramolecular endoperoxide bridge of ART (Fenton reaction), leading to the generation of carbon-centered radical molecules and reactive oxygen intermediates (ROIs). These free radical intermediates may be implicated in a cascade of alkylation reactions of specific *Plasmodium* proteins, causing damage to membrane-containing structures in particular (i.e. plasma membrane, endoplasmic reticulum, and nuclear membrane), and, ultimately, death of the parasite (reviewed by Meshnick, 2002). A similar mechanism might be responsible for the antiviral activity of ART considering the fact that ROIs are regulators of the activity of cellular redox-sensitive factors such as NF-κB. ROIs can enhance the signal transduction pathways for NF-κB activation in the cytoplasm and translocation into the nucleus, whereas the DNA-binding activity of oxidized NF-κB is significantly diminished (Kabe et al., 2005). In fact, we were able to detect a dimished DNA-binding activity of NF-κB in the presence of ART in HCMV-infected cells (Efferth et al., 2002).

In addition to the anti-malaria activity and antiviral activity, ART also exhibits profound cytotoxic activity towards tumor cells, whereas it remains non-cytotoxic to normal cells (Woerdenbag et al., 1993; Efferth et al., 2001, 2003, 2004; Singh and Lai, 2001; Dell'Eva et al., 2004). Analogous to the anti-malaria activity, ferrous iron might also play a prominent role in the cytotoxic activity of ART towards tumor cells. Indeed, intracellular iron concentrations are higher in tumor cells than in normal cells (Shterman et al., 1991). Cellular uptake and internalization of iron is mediated by binding of holo-transferrin (Tf)-iron complexes to the transferrin receptor (TfR), an integral membrane glycoprotein expressed in various human tissues (Gatter et al., 1983). Interestingly, proliferating and malignant cells were found to express higher levels of TfR on their cell surface (Faulk et al., 1980; Sutherland et al., 1981; Trowbridge and Omary, 1981). Furthermore, proliferating cells (particularly tumor cells) internalize high amounts of ferrous iron through internalization of Tf via the TfR pathway (Kollia et al., 2001; Singh and Lai, 2001; Yang et al., 2001; Sadava et al., 2002). The increased intracellular iron concentrations in tumor cells may thus render these cells susceptible to treatment with ART.

To investigate the antiviral action of ART, either in the presence or absence of ferrous iron, we set out to test its activity on the replication of human and animal cytomegaloviruses in vitro and in vivo. Regarding the therapeutic application of ART in the future, we investigated whether or not the antiviral activity of ART is additive to that of the conventional anti-HCMV drugs.

2. Materials and methods

2.1. Cell culture and virus

Primary human foreskin fibroblasts (HFF) and primary rat embryo fibroblasts (REF) were cultivated in MEM containing 5% (v/v) fetal calf serum. Cultivation of cells was restricted to passage numbers below 20. Human cytomegalovirus (HCMV) AD169-GFP (Marschall et al., 2000) and rat cytomegalovirus (RCMV) strain Maastricht (Bruggeman et al., 1982, 1983, 1985) were grown in HFF or REF, respectively, and virus titers were determined by either GFP expression (GFP-TCID) or plaque formation (PFU), respectively. In case of infection experiments, cells were grown overnight in 12-well or 6-well plates to subconfluent layers and inoculated with virus in volumes of 500 or 1000 μl per well for 90 min at 37 °C, after which inoculi were removed and cells were cultivated in fresh medium.

2.2. RNA isolation and RT-PCR

Semi-quantitative reverse transcriptase polymerase chain reaction was performed using the Titan One Tube RT-PCR System (Roche) and specific primer pairs for the amplification of TfR or GAPDH, respectively (TfR-sense, TATCTGCTATGGGACTATTGC; TfR-antisense, CGCCACATAACCCCCAGGATT; 698GAP5, GTACGTCGTGGAGTCCACTG; 698GAP3 TCCACCACCCTGTTGCTGTA). PCR cycling parameters were as follows: 10 cycles of 94 °C for 15 s, 50 °C for 30 s and 68 °C for 45 s, followed by additional 20 cycles, respectively, with 5 s elongation of each polymerization step at 68 °C per cycle. RNA template concentration was 2 μg per reaction.

2.3. Antiviral compounds

Compounds were purchased from the following companies: ganciclovir (GCV, Cymevene; Syntex-Arzneimittel/Roche, Germany), cidofovir (CDV, Vistide; Pharmacia & Upjohn S.A., Luxembourg), foscarnet (FOS, PFA; Sigma-Aldrich, Germany), artesunate (ART; Saokim Ltd., Hanoi, Vietnam), artemisinin (ARS; Sigma-Aldrich, Germany), holo-transferrin (Tf; Sigma-Aldrich, Germany), Ferrosanol™ (Fe, iron (II) glycine sulfate; SANOL GmbH, Monheim, Germany) and Taxol (Paclitaxel; Calbiochem/Merck, Germany). Stocks were prepared in aqueous solution or in DMSO (ART, Taxol) and aliquots were stored at −20 °C.

2.4. HCMV GFP-based antiviral assay

HFFs were grown in 12-well plates and used for infection with AD169-GFP virus at a multiplicity of GFP-TCID 0.25 (i.e. 25% GFP-forming tissue culture infectious dose at 7 days post infection). All infections were performed in duplicate. Then, infected cell layers were incubated with 2.5 ml of MEM containing 5% (v/v) fetal calf serum and, optionally, antiviral compounds. Infected cells were incubated at 37 °C in a 5% CO_2 atmosphere for 7 days. For GFP quantification, cells were lysed and lysates from each well were divided into two independent samples for further processing. Samples were centrifuged to remove cell debris and subsequently transferred to an opaque 96-well plate for automated measurement of GFP signals in a Victor 1420 Multilabel Counter (Perkin Elmer Wallac GmbH, Freiburg, Germany; Marschall et al., 2000).

2.5. Flow cytometry analysis (FACS)

HFFs were preincubated with 15 μM ART or an equivalent volume of the solvent (DMSO) 2 h before infection. Infection with HCMV AD169-GFP was performed in the presence of ART (15 μM) or DMSO. After adsorption of the virus (90 min), cells were cultivated with fresh culture medium containing ART or DMSO, respectively. At 72 h post infection, cells were fixed with 3% formaldehyde for 15 min at room temperature. For antibody staining, cells were first Fc-blocked with 1 mg/ml Cohn-II fraction for 15 min on ice, then stained with an antibody against Thy-1 (mouse IgG1-phycoerythrin (PE)-conjugated antihuman CD90, dilution 1:50; BD PharMingen, San Diego, CA, USA) for 25 min on ice. To assess background staining, cells were treated with a PE-conjugated IgG1-isotype control antibody (BD PharMingen). Thy-1 expression was determined by FACS analysis using a Becton Dickinson FACScalibur and CellQuest software (BD PharMingen).

2.6. Plaque reduction assay

HFFs or REFs were infected with HCMV or RCMV, respectively, for 90 min at 37 °C after which virus was removed and the cell layers were rinsed with PBS. Overlays of MEM containing 5% (v/v) fetal calf serum and 0.3% (w/v) agarose, optionally supplemented with antiviral compounds, were added to the wells. After incubation at 37 °C for 8–12 days, overlays were removed for staining of plaque formation with 1% crystal violet.

2.7. Cytotoxicity assay

HFF cells were cultivated in 48-well plates and antiviral compounds were incubated in the medium at 37 °C for 7 days. Measurement of the lactate dehydrogenase (LDH) activity in the culture medium was performed by the use of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Samples of 50 μl of culture media were incubated in 96-well plates with 50 μl of substrate mix for 30 min at room temperature in the dark. Thereafter, 50 μl of stop buffer was added and the color reaction was quantified by the use of an ELISA reader (OD 490 nm).

2.8. Replication of RCMV in vivo

Male specific-pathogen-free (spf) Lewis/M rats (Central Animal Facility, Maastricht University, Maastricht, The Netherlands) were kept under standard conditions (Stals et al., 1990). All experimental protocols mentioned in this paper were approved by the Maastricht University Animal Experiments Committee and were consistent with the Dutch Laboratory Animal Care Act. Twenty-four immunosuppressed rats (7 weeks old, 250–300 g of body weight) were assigned randomly to four groups. Rats were immunosuppressed by exposure to 5 Gy of total body Röntgen irradiation 1 day before infection, as described previously (Stals et al., 1990). Rats in two of the four groups received 50 mg/kg Ferrosanol^{TM} (Fe) daily via their drinking water, starting at 7 days before infection. Two days before infection, oral ART (50 mg/kg) prepared from 50-mg tablets and dissolved in 2.5% sodium carbonate solution, was administered daily to rats in both these groups. Subsequently, one group was mock infected, whereas the other was infected with RCMV. The third group of six rats did not receive Fe, but was only administered oral ART (50 mg/kg/day) and infected with RCMV, while the last group only received RCMV. Infection was carried out intraperitoneally with 1×10^6 plaque-forming units (PFU) of RCMV. Virus stocks that were used for inoculation were derived from salivary gland extracts from RCMV-infected rats, as described previously (Bruggeman et al., 1982, 1983). A separate, similar experiment consisted of only two groups of five 7-weeks-old, immunosuppressed rats. One group was administered Fe and ART (both at 50 mg/kg/day) as well as infected with RCMV, whereas the other group was infected with RCMV but not treated with Fe and ART. Rats were sacrificed at indicated time points. Their internal organs (salivary glands, spleen, kidney, liver, lung, thymus and blood) were collected and subjected to both plaque titer determination and real-time, quantitative PCR, as described earlier (Kaptein et al., 2003, 2004). The plaque assay and PCR data were statistically analyzed by applying a Mann–Whitney test using SPSS (SPSS International BV). P values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. ART inhibits HCMV replication in vitro: efficacy of antiviral activity is increased by addition of soluble holo-transferrin and ferrous iron

Previously, we reported that ART acts as an inhibitor of HCMV replication in human primary fibroblasts (Efferth et al., 2002). In this study, IC_{50} values of ART were determined as 3.7 ± 0.1 μM for HCMV AD169 and 3.9 ± 0.6% for HCMV AD169-GFP, respectively. Thus, measurement of the antiviral activity performed by plaque reduction assay (AD169) and GFP-based antiviral assay (AD169-GFP; Marschall et al., 2000) produced similar results. As analyzed in parallel, the inhibitory activity of GCV was in the same micromolar range as that of ART (Fig. 1A).

Other inhibitory activities of ART and related artemisinins, such as anti-malaria and anti-cancer activity, are largely dependent on high intracellular concentrations of ferrous iron (Fe^{2+}) that can be increased by stimulating the holo-transferrin (Tf) pathway of iron uptake (Cumming et al., 1997; Singh and Lai, 2001; Efferth et al., 2004). In order to study whether iron is capable of increasing the anti-HCMV activity of ART via the Tf-TfR pathway, we first addressed the question whether HCMV-permissive cells express the transferrin receptor (TfR). RT-PCR was performed to detect TfR-specific mRNA in total RNA isolated from human primary fibroblasts (HFFs), U373 cells and human PBMCs as a control. As shown in Fig. 1B, TfR-specific transcripts could easily be detected for all cells analyzed, indicating that in these cells the transferrin receptor is readily expressed. Subsequently, in order to address the iron/transferrin issue, HCMV replication was investigated in infected fibroblasts

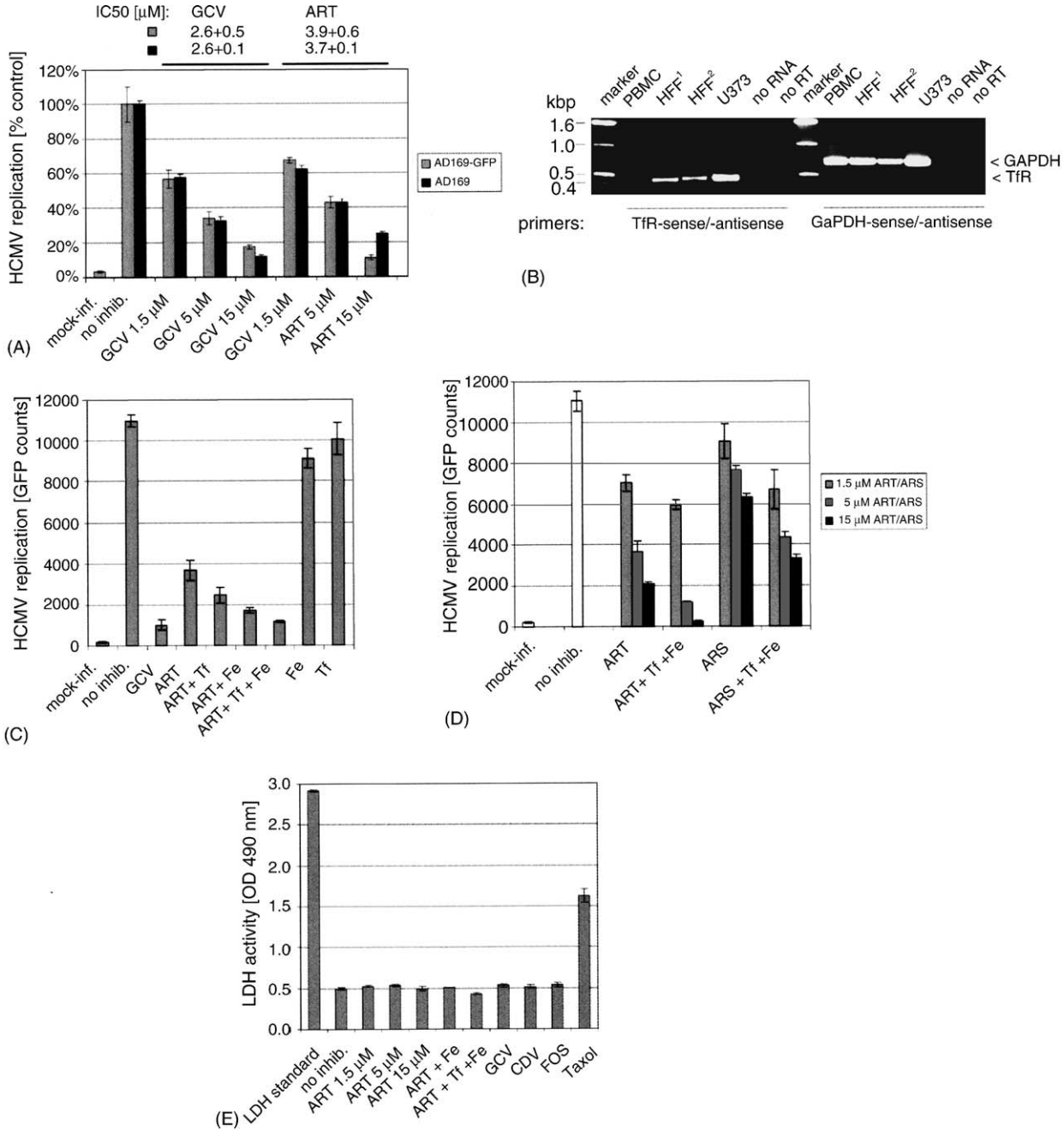

Fig. 1. Enhanced inhibition of HCMV replication in vitro by a combination of ART with Fe and/or Tf. (A) The anti-HCMV activity of ART and GCV was analyzed in parallel using GFP-based antiviral assay (AD169-GFP) and plaque reduction assay (AD169). HFFs were cultivated in 12-well plates and used for infection with either of the two HCMVs. Drugs were added immediately after virus adsorption to the culture media and incubated for 7 days before HCMV replication was quantified. (B) Expression of TfR in various cell types was analyzed by RT-PCR. Primers with specificity for TfR (left panel) or GAPDH (used as a positive control, right panel) were chosen to obtain RT-PCR products as indicated. PBMC, peripheral blood mononuclear cells; $HFF^{1/2}$, low and high passages of primary human foreskin fibroblasts; U373, astrocytoma cells; no RNA, control without template; no RT, control without reverse transcriptase. (C) and (D) HFFs were cultivated in 12-well plates and used for infection with HCMV AD169-GFP (GFP-$TCID_{50}$ 0.25). Drugs were added immediately after virus adsorption to the culture media and incubated for 7 days. Virus replication was quantified by automated GFP fluorometry. (E) HFFs were cultivated in 48-well plates, incubated with the compounds indicated for 7 days, and used for the determination of LDH acitivty in the culture media as a marker of cytotoxicity. Each panel in (A), (C), (D) and (E) shows the data for infections/compound incubations in duplicate plus GFP/plaque quantification in duplicate. Mock-inf., uninfected; no inhib., infected an incubated with DMSO solvent alone; GCV/ART/ARS/Tf/Fe/CDV/FOS/Taxol, infected and/or incubated with the following concentrations of ganciclovir (as indicated; 20 μM in C and E), artesunate (as indicated; 5 μM in C), artemisinin (as indicated), holo-transferrin (1 mg/ml), Ferrosanol™ (1 μg/ml), cidofovir (2 μM), foscarnet (200 μM) and Taxol (1 μM).

that were treated with ART (at a concentration of 5 μM), either in the presence or absence of Ferrosanol™ (Fe) and/or holotransferrin (Tf). As anticipated, administration of ART caused a clear decline in HCMV replication in vitro (Fig. 1C). This decrease was even more pronounced when HCMV-infected cells were treated with either ART plus Tf or ART plus Fe. Importantly, HCMV replication was most strongly inhibited when ART was administered in combination with both Fe and Tf (Fig. 1C). Fe alone had a marginal virus-suppressing effect. As shown in Fig. 1D, the antiviral effect of ART is concentration-dependent. At each concentration of ART used, an additional reduction of viral replication was seen when the drug was administered in combination with Fe and Tf. A concentration-dependent decline in HCMV replication was also observed when using an ART-related compound, artemisinin (ARS; Fig. 1D). However, the overall inhibitory effect of ARS, either or not combined with Tf plus Fe, was lower than that of ART, indicating that ART is the more effective HCMV-inhibiting agent. Thus, the antiviral activity of ART and ARS can be enhanced by coadministration of drugs that either directly or indirectly increase intracellular ferrous iron concentrations.

In order to address the question of drug-induced cytotoxicity, the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) was performed. No cytotoxicity on primary human fibroblasts was observed with any of the drugs or combinations of drugs analyzed (Fig. 1E; for additional data see Efferth et al., 2002). Taken together, the anti-HCMV activity is specific and devoid of cytotoxicity in the range of concentrations showing antiviral activity.

3.2. ART prevents the HCMV-induced downregulation of the cellular adhesion molecule Thy-1 (CD90)

Human cytomegalovirus is known to modulate the cell surface expression of several adhesion molecules like ICAM-1, CD40 or Thy-1 (CD90) and this appears to contribute to the pro-inflammatory effects of HCMV infection (Einsele et al., 1994; Lemström et al., 1995; Steinhoff et al., 1995; Koskinen et al., 1996; Yilmaz et al., 1996; Martelius et al., 1998; Waldman et al., 1998; Lautenschlager et al., 1999; The et al., 2001). Since established antiviral drugs like GCV and CDV are not able to prevent this pro-inflammatory activity, we investigated whether ART plus stimulating agents might inhibit the effect of HCMV on the expression of cell–surface molecules (Maisch et al., 2002; Craigen and Grundy, 1996; Cinatl et al., 2000). Recently, we demonstrated the modulation of Thy-1 (CD90) expression in HCMV-infected HFFs (Leis et al., 2004). Therefore, we investigated the influence of ART on the Thy-1 down-regulation on the surface of HCMV (AD169-GFP)-infected HFFs by flow cytometry. ART inhibited the down-regulation of Thy-1 (Fig. 2): 29.8% of the infected, GFP-positive cells showed a decreased level of Thy-1 expression versus mock-infection (0.2%), while in the presence of ART (15 μM) the decreased level of Thy-1 expression was only 13.0% (mock 0.0%). Thy-1 down-regulation could be blocked even more efficiently when ART was applied in combination with Tf and Fe (data not shown). Thus, ART not only inhibits HCMV replication but also reduces HCMV-induced modulation of cell surface Thy-1 expression on human fibroblasts. This finding confirms the strong antiviral potential of ART.

3.3. Antiviral effects of ART applied in combination with conventional drugs of HCMV therapy are additive

To investigate the influence of the ART treatment on the efficacy of conventional anti-HCMV drugs, HCMV-infected cells were administered a combination of ART and either one of the conventional drugs. Using intermediate concentrations of GCV (1 μM), CDV (0.1 μM) and FOS (100 μM), the combined treatment with ART (at a concentration of 1.5 and 5 μM, respectively) led to distinct, additive inhibitory effects on HCMV replication (Fig. 3). Antagonistic effects were not observed. The additive effect of ART with GCV could be increased by the addition of Fe (1.0 μg/ml; data not shown). These findings illustrate that the mode of action of ART is different from that of GCV, CDV and FOS, which all act on the stage of viral DNA synthesis. Thus, a clinical regimen based on a combination of ART with conventional antiviral agents for the treatment of HCMV disease seems highly promising.

3.4. ART shows antiviral activity against human and animal cytomegaloviruses

The inhibitory effect of ART on human CMVs is not limited to laboratory strain AD169, since several virus strains tested, such as Towne and clinical isolates, particularly those possessing resistance against GCV and/or CDV, are similarly sensitive to ART (Efferth et al., 2002). Interestingly, other herpesviruses

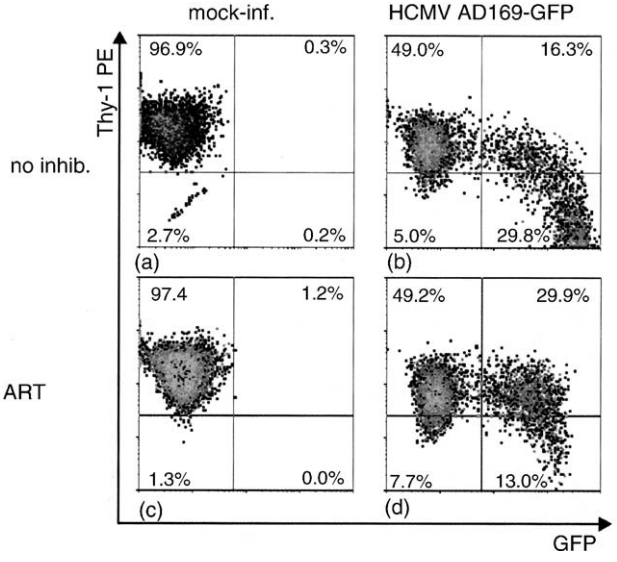

Fig. 2. Inhibition of Thy-1 modulation on HCMV-infected cells. HFFs were infected with HCMV AD169-GFP or mock infected and treated with ART (15 μM) or solvent DMSO alone (no inhib.). At 72 h post infection, cells were fixed and analyzed by flow cytometry (FACS). Virus replication was quantified by GFP measurement, Thy-1 expression by PE staining (antibody labelling). Percentage of positive cells is indicated for each signal.

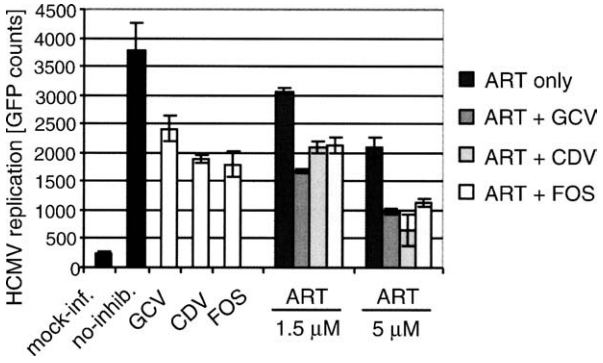

Fig. 3. Combined treatment of HCMV-infected cells with ART and conventional antiviral drugs. HFFs were cultivated in 12-well plates and used for infection with HCMV AD169-GFP (GFP-$TCID_{50}$ 0.25). Single drugs or combinations of drugs were added immediately after virus adsorption to the culture media and incubated for 7 days. Virus replication was quantified by automated GFP fluorometry. Each panel shows the data for infections in duplicate plus GFP quantification in duplicate. Mock-inf., uninfected; no inhib., infected and incubated with DMSO solvent alone; GCV/CDV/FOS/ART, infected and incubated with ganciclovir (1 μM), cidofovir (0.1 μM), foscarnet (100 μM) and/or artesunate (as indicated).

are also subject to the inhibitory effect of ART. Previously, we described the efficient inhibition of the in vitro replication of a clinical isolate of herpes simplex virus (HSV-1) (Efferth et al., 2002). Furthermore, inhibition of Epstein–Barr virus (EBV) by ART was detected using a reporter system for the quantification of lytic reactivation from viral latency (Marschall et al., 1993; data not shown). Importantly, antiviral activity of ART was also detectable for rat cytomegalovirus (RCMV) in REF (Fig. 4). In addition, combined treatment with Fe

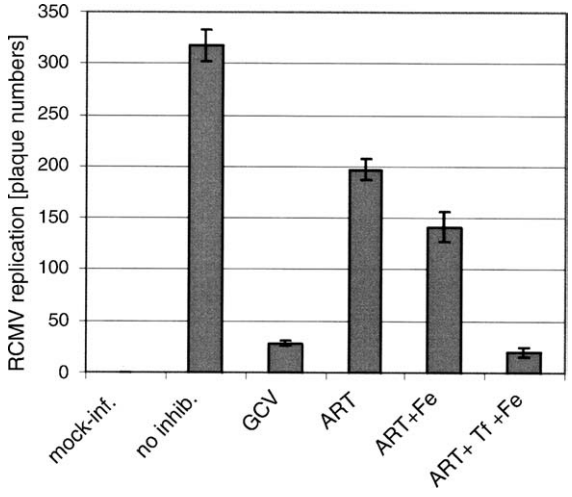

Fig. 4. Inhibition of RCMV by ART in vitro. REFs were cultivated in 12-well plates and used for infection with RCMV strain Maastricht (MOI 0.01). After virus adsorption, cells were overlayed with agar-media including the drugs indicated, incubated for 10 days and analyzed by staining of plaques. Each panel shows the data for infections in duplicate plus microscopic counting of plaques in duplicate. Mock-inf., uninfected; no inhib., infected and incubated with DMSO solvent alone; GCV/ART/Fe/Tf, infected and incubated with ganciclovir (20 μM), artesunate (5 μM), Ferrosanol™ (1 μg/ml) and/or holotransferrin (1 mg/ml).

and/or Tf increased the anti-RCMV effect of ART. Comparing RCMV with HCMV, however, the efficiency of inhibition was lower (RCMV $37.9 \pm 3.2\%/55.4 \pm 4.5\%/93.6 \pm 1.4\%$ inhibition versus HCMV $81.2 \pm 0.9\%/96.4 \pm 0.4\%/97.6 \pm 0.4\%$ inhibition, when treated with $ART_{15\,\mu M}/ART_{15\,\mu M}$ plus $Fe_{1\,\mu g/ml}$/ $ART_{15\,\mu M}$ plus $Fe_{1\,\mu g/ml}$ plus $Tf_{1\,mg/ml}$, respectively; primary data, see Figs. 4 and 1D). Taken together, the anti-CMV activity of ART is not solely restricted to HCMV and it seemed likely that similar antiviral effects would also be detectable in an animal model.

3.5. *Effect of ART on the replication of RCMV in immunocompromised rats*

To assess whether treatment with oral ART affects the dissemination and replication of RCMV in vivo, the following animal experiment was performed. Oral ART was administered daily, either with or without treatment with Ferrosanol™ (Fe), to immunosuppressed, RCMV-infected rats (Fig. 5). Rats that received both ART- and Fe-treatment as well as mock-infection were regarded as negative controls. Accordingly, RCMV-infected rats that were not administered ART and Fe, were considered positive controls. At day 3 and 15 pi, rats were sacrificed and dissected, after which their internal organs were analyzed for the presence of virus and viral DNA by plaque assay and real-time, quantitative PCR, respectively. At day 3 pi, the spleen from RCMV-infected rats (irrespective of treatment with ART and/or Fe) was demonstrated to contain the highest viral DNA load (data not shown). This finding is in agreement with previous reports (Kaptein et al., 2003, 2004). Similarly, of all internal organs and tissues examined, the spleen was found to contain the highest levels of infectious virus at day 3 post infection (pi) (data not shown). However, neither the viral DNA loads, nor the virus titers differed significantly in the spleen as well as any of the other internal organs examined between treated and untreated rats infected with RCMV (data not shown). Importantly, at day 15 pi, a significant difference was found between RCMV-infected rats treated with ART and Fe on the one hand and untreated, RCMV-infected rats on the other ($P < 0.05$; Fig. 6). Although viral DNA loads could be detected in salivary gland samples of RCMV-infected rats treated with ART and Fe, the viral DNA levels were significantly lower than those of untreated, RCMV-infected rats (Fig. 6A and C). Salivary glands from RCMV-infected rats treated with ART and Fe contained a mean of 1.9×10^3 viral DNA copies per microgram of tissue, whereas the salivary glands from untreated, RCMV-infected contained a mean of 1.5×10^4 viral DNA copies per microgram tissue. This difference was even more reflected by the levels of infectious virus in the salivary glands from treated and untreated rats: salivary glands from RCMV-infected rats treated with ART and Fe were found to contain approximately 20-fold lower levels of infectious virus than those from untreated, RCMV-infected rats ($P < 0.05$; Fig. 6B and D). Therefore, these data indicate that ART treatment, in combination with Ferrosanol™ treatment, inhibits RCMV dissemination to or infection of the salivary glands and that ART has a significant antiviral effect in vivo.

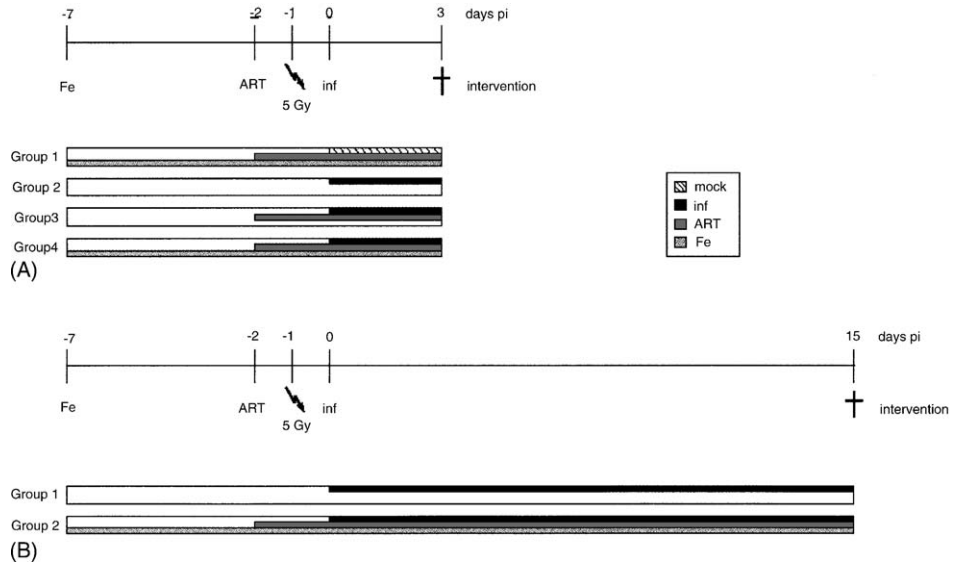

Fig. 5. Flow-charts of in vivo experiments. Rats were assigned randomly to several intervention groups as indicated. Ferrosanol™ treatment was started 7 days before infection (Fe; light grey boxes), artesunate treatment 2 days before infection (ART; dark grey boxes). The period of RCMV infection is indicated by black boxes, the mock infected control by a hatched box. Rats were sacrificed at day 3 (A) and 15 (B) after infection and their internal organs were investigated for the presence of viral DNA and infectious virus. All rats were immunosuppressed 1 day before infection (indicated by broken arrows).

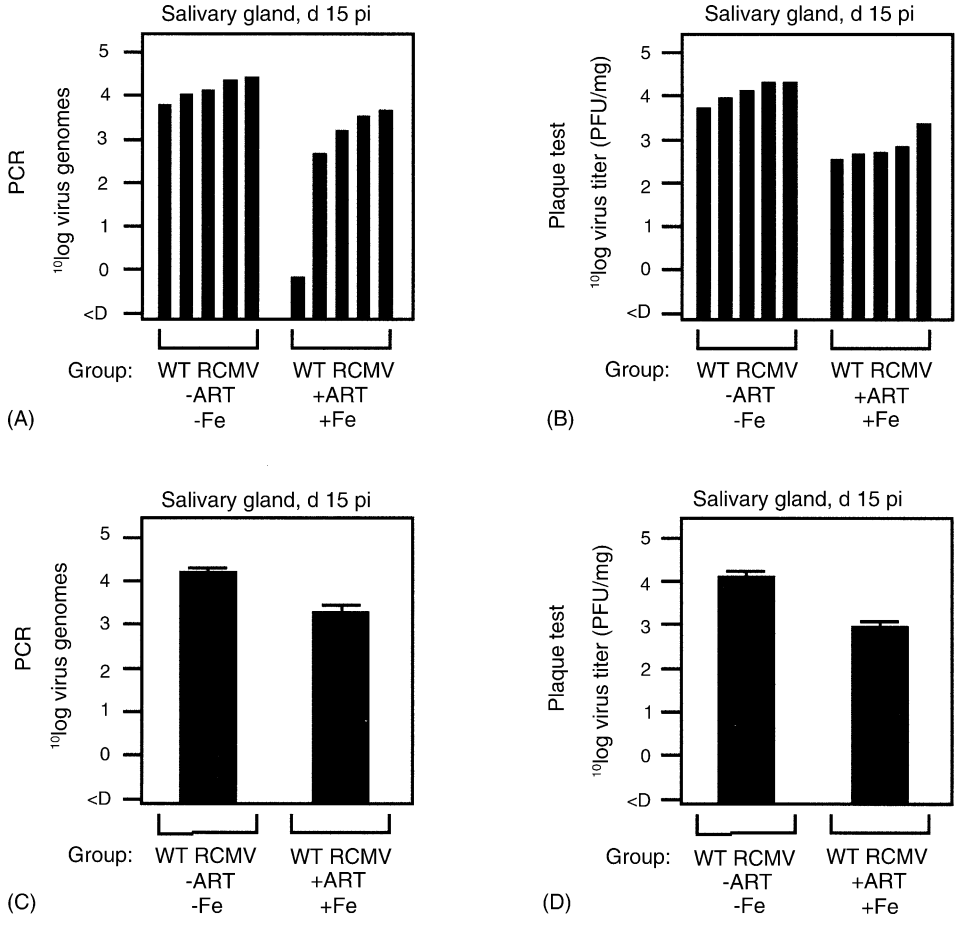

Fig. 6. Dissemination of RCMV to the salivary glands of rats, either with or without treatment with artesunate (ART) and/or Ferrosanol™ (Fe), at day 15 post infection (pi). Amount of viral genome copies in salivary glands from infected rats (A and C). Virus titers in the salivary glands from infected rats (B and D). In the upper graphs (A and B) each bar represents the result from a single rat ($n = 5$ rats per group). In the lower graphs (C and D) the results are expressed as the mean ± S.E.M. ($n = 5$ rats per group). (D) Below detection level (for the plaque assay, 10^{-1} PFU/mg tissue; for PCR, 10^{-1} viral genome copies/mg tissue DNA).

4. Discussion

Previously we reported that ART, an anti-malaria drug with highly auspicious pharmacological properties, possesses antiviral activity. In vitro, ART displays a strong inhibitory effect against the replication of HCMV as well as herpes simplex virus type 1. Moreover, ART was shown to partially inhibit human immunodeficiency virus type 1, whereas inhibition of human influenza A virus was not detectable (Efferth et al., 2002). Here, we demonstrate that the antiviral activity of ART against CMV is not restricted to human strains, but also includes animal CMVs, in particular RCMV. An important feature seems the finding that increased intracellular iron concentrations in the presence of ART significantly enhance its anti-CMV activity. This enhancing effect was demonstrated by several observations: (i) treatment of CMV-infected fibroblasts with ART combined with ferrous iron (Ferrosanol^{TM}) and/or soluble Tf resulted in enhanced suppression of viral replication, (ii) the expression of a cell surface marker (Thy-1), which is associated with the pro-inflammatory effect of CMV infection and which is not affected by established antiviral drugs, is strongly influenced by ART treatment, (iii) the antiviral activity of ART against CMV could also be demonstrated in vivo by using the RCMV/rat model, and finally, (iv) the antiviral activity of ART is additive when applied in combination with conventional drugs such as GCV, CDV and FOS.

The latter finding might be particularly helpful in the treatment of HCMV disease inflicted by mutant viruses that are resistant to conventional antiviral drugs (reviewed by Alejo, 1999). GCV, CDV and FOS are all directed to an identical target of viral replication (i.e. DNA synthesis mediated by the viral DNA polymerase) and, consequently, cross-resistance conferred by polymerase-related mutations has frequently been reported (reviewed by Erice, 1999). The combination of drugs with different modes of action may delay the development of drug resistance in a clinical setting. Therefore, using an antiviral drug that targets an alternative pathway and does not interfere with activities of conventional antiviral drugs seems highly promising. Moreover, Ferrosanol^{TM} is a clinically approved medication and in practical use for many years. It is a safe drug without severe toxicity and can, hence, be safely combined with ART.

An important argument in favor of the usefulness of ART in antiviral therapy, particularly in combination therapy, is its lack of side effects. As demonstrated in a number of preclinical and clinical studies, ART treatment is not associated with severe side effects (reviewed by de Vries and Dien, 1996). Artemisinin and its derivatives may exert neurotoxic effects in animal models after prolonged exposure at high doses (Genovese et al., 2000; Nontprasert et al., 2000). However, this has never been observed in humans. Meta-analyses of malaria patients treated with artemisinins demonstrated that this drug class is safe (Adjuik et al., 2004; Ribeiro and Olliaro, 1998). One might expect a similarly high degree of safety for the potential use of ART as an antiviral drug. This seems particularly important in case of long-term antiviral treatment, e.g. upon HCMV reactivation in immunosuppressed transplant patients. With respect to pharmacokinetics, ART seems to be highly suitable for antiviral therapy in regimens of oral administration. The mean human plasma concentration of the main metabolite of ART (dihydroartemisinin, DHA) is 0.57 μg/ml after a single oral dose of 200 mg (Benakis et al., 1997). The plasma concentration of ART itself is relatively low due to the rapid and effective conversion of ART into DHA (Edlund et al., 1984; Li et al., 1998). Considering the fact that antiviral treatment (similar to malaria treatment) may be optimized through repeated administration, plasma concentrations are supposed to be within the effective range of HCMV inhibition. Tolerable doses in humans may very likely translate into clinical benefit in case of antiviral therapy, since during malaria therapy, ART concentrations up to 6.8 μM in the plasma were reported (equivalent to 2.6 μg/ml; reviewed by de Vries and Dien, 1996). The mean IC_{50} for inhibition of HCMV replication determined in this study is 3.8 μM. Moreover, the plasma concentration of ART in rats treated with a single oral dose of 150 mg was determined as C_{max} 0.71 μg/ml and the concentration of DHA was even higher (i.e. 2.04 μg/ml; Olliaro et al., 2001). In accordance to this, the in vivo data in the present study strongly suggest that the used dosing is effective for antiviral treatment. Thus, oral therapy with ART, possibly combined with Ferrosanol^{TM}, seems to be a beneficial option to the current therapies for treatment of HCMV disease.

Despite the many years of experience obtained with ART in medical use, relatively little is known about the antiviral mode of action of ART. A first concept was based on our recent finding that the block in HCMV replication already occurs prior to the synthesis of viral immediate early proteins. Furthermore, HCMV-induced cellular transcription factors, such as Sp1 and NF-κB, as well as the cellular signaling kinase phosphoinositide 3-kinase, required for the activation of Sp1 and NF-κB, were markedly reduced in ART-treated infected cells (Efferth et al., 2002). Therefore, the antiviral effect of ART seems to be mediated by an inhibition of cellular activation pathways that play an essential role in virus replication.

However, it should be mentioned that ART is not the sole candidate to be used in anti-HCMV therapy. Since the parental natural substance artemisinin possesses only relatively low anti-HCMV activity, as was also observed in this study, novel synthetic artemisinin derivatives are currently under investigation. Vennerstrom et al. (2004) reported that synthetic peroxide antimalaria drugs, such as trioxalanes 5-7, are very potent against malaria in vitro. Altogether, this study indicates that ART is a highly eligible candidate for future anti-HCMV treatment, preferably in combination with conventional antiviral drugs. Challenges for future studies will be (i) to investigate the anti-CMV potential of novel synthetic artemisinin derivatives, such as trioxalanes 5–7, and (ii) to elucidate the putative intracellular pathways by which ART as well as the other synthetic artemisinin derivatives affect cytomegalovirus replication in vivo.

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