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Short Communication

The unique antiviral activity of artesunate is broadly effective against human cytomegaloviruses including therapy-resistant mutants

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ABSTRACT

Current therapy options to treat infections with human cytomegalovirus face severe limitations leading to a continued search for novel drug candidates. Here, we describe novel characteristics of the strong antiviral potency of the drug artesunate. *In vitro* virus replication systems were applied to analyze a number of laboratory and clinically relevant strains of human cytomegalovirus. An inhibitory block at a very early stage of infection was demonstrated. Time-of-addition experiments indicated that the antiviral efficacy could be optimized when artesunate was applied as fractional doses consecutively added post-infection. Artesunate showed a clearly higher anti-cytomegaloviral activity than its parental drug artemisinin (approximately 10-fold) or other artesunate-related compounds. Mean IC_{50} values of artesunate for a variety of standard therapy-resistant virus mutants were within a 2-fold range compared to wild-type virus. Furthermore, a synergistic effect was identified when artesunate was combined with the mechanistically distinct antiviral compound maribavir. These findings point to unique antiviral properties of artesunate which may offer an advantage over standard antiviral therapy particularly in cases of drug resistance.

1. Short Communication

Human cytomegalovirus (HCMV) is the type species of the betasubfamily of *Herpesviridae* and is associated with severe forms of human diseases (Mocarski et al., 2007). Primary acute infection as well as lifelong persistence can lead to life-threatening clinical manifestations particularly in the immunocompromised host. High risk of HCMV disease is linked to transplant, tumor and AIDS patients as well as to neonates. The success of treatment with presently available anti-cytomegaloviral drugs is limited by adverse effects such as bone marrow and kidney toxicity, oral bioavailability and the selection of drug-resistant viruses (Lurain and Chou, 2010). Recently, an experimental pUL97 kinase inhibitor, maribavir (MBV), was developed (Biron et al., 2002), but unexpectedly failed in some late stage clinical trials (Marty et al., 2011; Strasfeld et al., 2010). Thus, the development of improved drugs and therapy regimens is in the focus of worldwide investigation (Marschall and Stamminger, 2009). Artesunate (ART), a semi-synthetic derivative of the natural product artemisinin, is commonly used in the treatment of severe malaria (Adjuik et al., 2004; Gomes et al., 2008). Antiviral activity of ART was first observed against HCMV *in vitro* and *in vivo* (Efferth et al., 2002; Kaptein et al., 2006). Other herpesviruses, such as rat and murine CMVs, human herpes virus type 6 (HHV-6), herpes simplex virus (HSV-1) and Epstein-Barr virus are also ART-sensitive (Efferth et al., 2002, 2008; Kaptein et al., 2006; Milbradt et al., 2009; Auerochs et al., 2011). Due to its high bioavailability and very limited side-effects, ART is a promising candidate for antiviral therapy. Recent case reports suggest that ART may be effective in treating severe HCMV infection (Wolf et al., 2011; Shapira et al., 2008). In the present study, a systematic analysis of the ART sensitivity (*in vitro* IC_{50}) of therapy-resistant virus mutants was performed.

For analyzing *in vitro* dosage characteristics, the antiviral activity of ART was quantitated using a GFP-based replication assay (HCMV AD169-GFP; Marschall et al., 2000; Rechter et al., 2006) with primary human foreskin fibroblasts (HFF) cultures infected at a GFP-MOI of 0.25 (Fig. 1A). When ART was incubated in the culture medium of HFFs immediately after the adsorption of HCMV (post), a strong antiviral effect was measured which was similar to the inhibitory effect of the reference drug ganciclovir (GCV). In contrast, when the drugs were only incubated prior addition of the virus (pre), neither ART nor GCV showed inhibition (Fig. 1B). Similarly, when ART was incubated before and during virus

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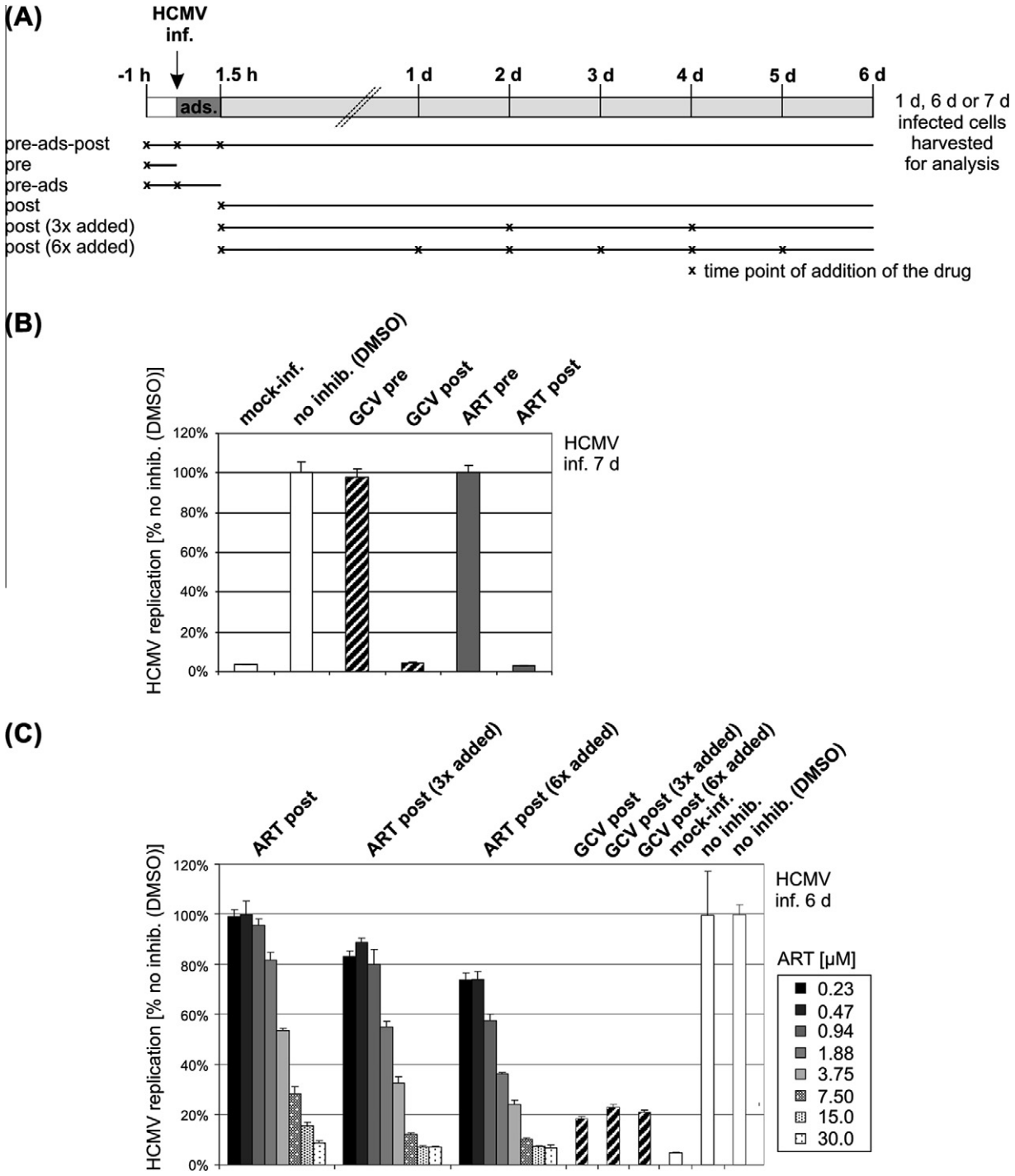

Fig. 1. The efficacy of antiviral activity of ART was characterized *in vitro* by comparing various time points of drug addition. Human primary fibroblasts (HFF) were infected with HCMV AD169-GFP or remained mock-infected. (A) Schematic representation of the schedule of infection and drug addition: *inf*., infection; *ads*, phase of virus adsorption (1.5 h); *pre*, preincubation of the drug; *pre-ads/pre-ads-post/post*, presence of the drug during different phases of infection; *3x/6x added*, additive fractional drug doses applied consecutively 3 or 6 times. (B) HCMV-infected cells were treated with ART or GCV at a concentration of 15 μM, harvested 7 day post-infection and used for a quantitation of viral load in the GFP-based replication assay. (C) HCMV-infected cells were treated with ART at the concentrations indicated or GCV as a control (7.5 μM), harvested 6 day post-infection and assayed as above. All determinations were performed in quadruplicate (mean values ± standard deviation).

adsorption, but thereafter was removed (pre-ads), no antiviral effect was detectable (Fig. 2, upper panel). Importantly, the antiviral efficacy could be optimized when ART was not applied as a single dose but as fractional doses consecutively added post-infection (i.e. either 3 times one-third or 6 times one-sixth of the final dose; Fig. 1C). The IC_{50} values for the three different modes of ART addition were 4.31 ± 0.17 μM (post), 2.34 ± 0.19 μM (post 3× added) and 1.29 ± 0.08 μM (post 6× added), respectively. In comparison to ART, an intermediate concentration of GCV (7.5 μM) effected an inhibition of HCMV replication of approximately 80%, but no comparable difference was noted for the three modes of drug addition (Fig. 1C, striped bars). This underlines the differences between ART and GCV in their antiviral modes of action (Efferth et al., 2002; Milbradt et al., 2009; reviewed by Efferth et al. (2008)).

Concerning the specific antiviral mechanism of ART, the synthesis of viral immediate early and early proteins during ART

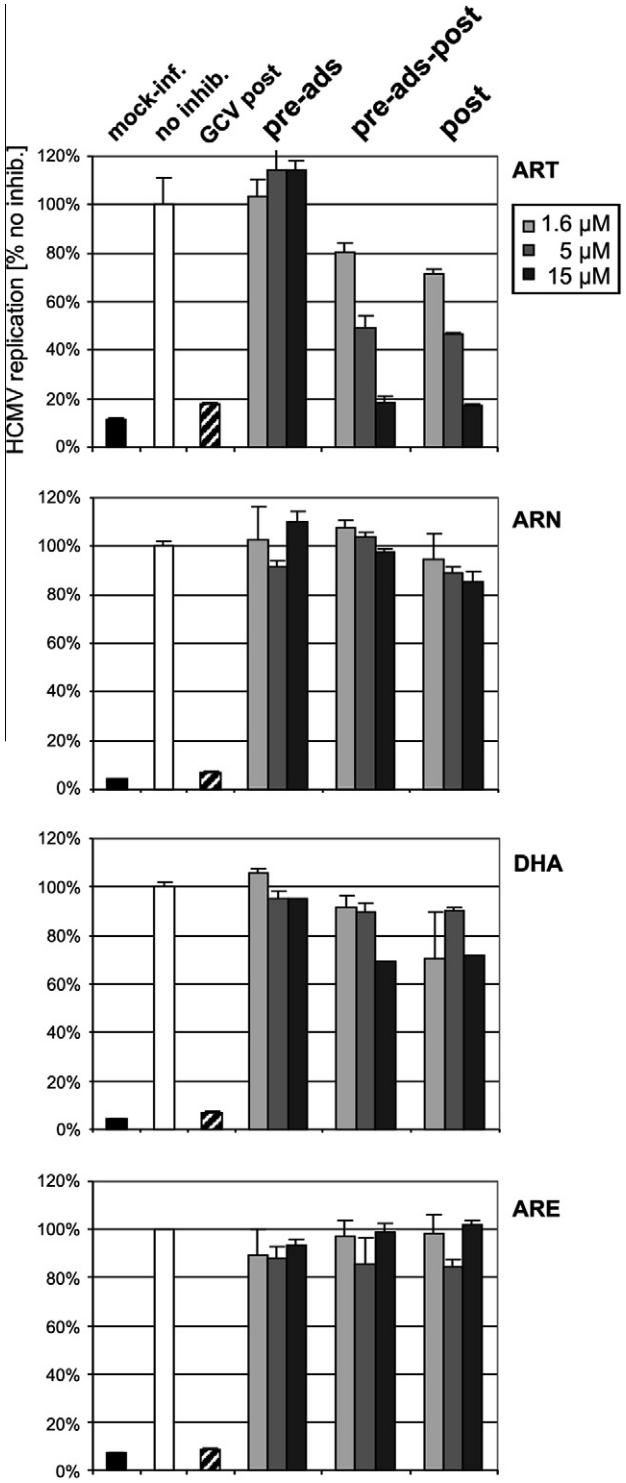

Fig. 2. Comparison of the anti-cytomegaloviral activity of ART with ART-related compounds. HFFs were infected with HCMV AD169-GFP or remained mock-infected. Cells were treated with ART, ARN, DHA or ARE at the concentrations indicated and assayed as described for Fig. 1.

treatment of HCMV-infected HFFs was analyzed and compared to data published previously (Efferth et al., 2002; Arav-Boger et al., 2010). For this purpose, HFF cultures (2.25×10^5 cells/well in a 12-well plate) were infected with HCMV AD169-GFP (MOI 0.25–0.5) and treated with ART under various conditions. Total cellular extracts were harvested 1 day post-infection and used for protein detection by Western blot analysis (Fig. S1; Auerochs et al., 2011; MAb-IE1p72, monoclonal IE1, and PAb-IE2p86, anti-pHM178, both derived from laboratory repository of T.S.; MAb-UL44, BS510, kindly provided by Bodo Plachter, Univ. Mainz, Germany; MAb-β-actin, AC-15, Sigma–Aldrich). Most efficient antiviral activity of ART was measured when the drug was present continuously, starting 1 h before infection until 1 day post-infection (pre-ads-post; Fig. S1A, lane 4). Under these conditions, a clear down-regulation of viral immediate early protein synthesis was detectable, resulting in an effective block with consequences on later steps of viral replication. Moreover, the block in protein synthesis could be enhanced when the treatment with ART was performed in an additive way by applying three fractional doses (Fig. S1B). Thus, an optimization of the antiviral activity of ART is possible by repeated treatment.

As a next step, the question was addressed whether the main intracellular metabolite of ART, dihydro-artemisinin (DHA) or ART-related sesquiterpene compounds, such as the naturally occurring parental drug artemisinin (ARN) and semisynthetic derivative artemether (ARE) show anti-cytomegaloviral activity. Stocks of antiviral compounds were prepared in DMSO (ART, Soakim Ltd, Hanoi, Vietnam and Dafra Pharma, Turnhout, Belgium; ARN, Sigma; DHA, Sigma; ARE, Dafra Pharma) or aqueous solution (GCV, Cymeven, Syntex-Arzneimittel/Roche; MBV, GlaxoSmithKline/ViroPharma) and stored at −20 °C. Compared to ART, none of the three related compounds was similarly effective, neither under post nor under pre-ads or pre-ads-post-conditions (Fig. 2). While ART was highly active (post- and pre-ads-post), the ART-related drugs ARN, DHA and ARE showed very little or no anti-cytomegaloviral activity. This finding indicates that the specific side-chain moiety of ART, $OC(O)CH_2CH_2COOH$, which is not present in the other compounds, might have an impact on its antiviral activity as discussed before (Milbradt et al., 2009; Efferth et al., 2008). It should be mentioned that a recent report by Arav-Boger et al. (2010) presented data which are partly in conflict with our findings. The report, based on slightly different experimental systems compared to the present study, described similar anti-HCMV efficacy for ART and ART derivatives (including ARN and ARE). In our hands, a theoretical extrapolation of the data in Fig. 2 (ARN, right columns) suggested an IC_{50} of 51.94 ± 0.01 μM for ARN under conditions of exclusive post-incubation, which means an approximately 10-fold higher value than ART. It remains speculative whether the methodical differences between the two studies (HCMV strains AD169 versus Towne, reporter assays GFP/SEAP versus luciferase, or others) might have contributed to inconsistent results.

Next, the question was addressed whether ART is similarly active against a panel of therapy-resistant HCMV mutants. The SEAP yield reduction assay was used to determine drug sensitivities of the AD169-derived strain T2211 containing a secreted alkaline phosphatase (SEAP) reporter gene and its derived recombinant UL27, UL54 and UL97 mutant viruses, which have previously been tested for susceptibility or resistance to GCV, MBV and other standard antivirals (Chou et al., 2005, 2007; Chou, 2009, 2011; Chou and Marousek, 2008). Rows of 24-well plates containing confluent monolayers of $\sim 2 \times 10^5$ fibroblasts (HEL or HFF) were inoculated with virus stock at a MOI of 0.01–0.03 in a volume of 0.3 ml, incubated for 90 min without drug, then cultured for 6 days under 1 ml of medium containing no drug (control), or serial 2-fold dilutions of ART up to 8 or 16 μM. Culture supernatant was sampled at day 6 for chemiluminescent assay to determine the drug concentration required to reduce supernatant SEAP activity by 50% (IC_{50}) of the no-drug control well. The definition of drug resistance was >2-fold elevation of IC_{50} value over a matching baseline control strain without the stated mutation. Here, the previously constructed SEAP reporter strains containing mutations that confer resistance to ganciclovir (GCV), foscarnet (FOS), cidofovir (CDV) or maribavir (MBV) were tested against ART. Results shown in Table 1 indicate a comparable activity of ART in HFF and HEL fibroblasts, with an overall mean IC_{50} for all

Table 1
Artesunate IC_{50s} in HCMV-infected HEL versus HFF.

Strain	Gene	Mutation	Resistance phenotype[a]	Artesunate in HEL cells			Artesunate in HFF cells		
				Mean IC_{50}[b]	Std. Dev.	N[c]	Mean IC_{50}[b]	Std. Dev.	N[c]
T2211		wt	None	3.26	1.36	23	2.76	0.58	33
T3020	UL27	E22stop	MBV-low	4.40	0.93	10	4.05	1.40	9
T2890	UL97	V353A	MBV-mod	3.84	1.61	12	2.70	0.76	10
T2264	UL97	L397R	MBV-hi	3.40	1.55	12	2.41	0.84	10
T2758	UL97	T409M	MBV-mod	4.13	1.42	10	2.62	0.47	11
T2923	UL97	H411L	MBV-mod	4.71	2.09	14	3.02	0.54	10
T2259	UL97	M460V	GCV	3.52	1.65	18	2.87	0.58	16
T2255	UL97	A594V	GCV	4.41	2.15	12	4.93	0.98	16
T2260	UL97	L595S	GCV	6.25	1.22	10	3.37	0.98	18
T2293	Pol	N408K	GCV-CDV	4.76	1.44	15	3.58	0.89	12
T3267	Pol	F412L	GCV-CDV	4.60	1.16	9	2.57	0.34	10
T3005	Pol	P522A	GCV-CDV	5.12	1.22	12	2.60	0.47	10
T3430	Pol	E756K	FOS	4.51	1.62	12	3.82	0.76	11
T3417	Pol	V781I	FOS	5.01	0.99	7	3.24	0.93	9
T2417	Pol	A809V	FOS	4.20	2.60	16	2.49	0.52	24
T2420	Pol	G841A	FOS	4.26	1.61	7	3.30	0.56	9
T2222	Pol	981-2del	GCV-FOS-CDV	4.62	1.91	9	2.62	0.75	10
T3429	Pol	A987G	GCV-CDV	3.69	1.16	8	2.61	0.43	8
All strains				**4.37**			**3.09**		

[a] Principal resistance phenotype of the mutation. GCV, ganciclovir; FOS, foscarnet; CDV, cidofovir; MBV, maribavir.
[b] Mean value (μM) of the number of replicates shown.
[c] Number of replicates.

strains tested found to be in the 3–4 μM range, consistent with the data in Fig. 2. There was a narrower range of IC_{50} values (and lower standard deviations) in the HFF cultures, suggesting a lesser effect of slight variations in cell culture conditions. All of the drug-resistant mutants had IC_{50} values for ART that were not more than 2-fold increased over the wild-type control or the overall mean IC_{50} value, in both fibroblast cultures. Assays were performed in two fibroblast types because for some drugs, notably MBV, there is a great difference in the IC_{50} values when determined in HFF versus HEL cultures (Chou et al., 2006). In contrast to ART, IC_{50} values for ARN in this assay system (HEL cultures) were approximately 10-fold higher for both the wild-type control strain T2211 (mean IC_{50} of 37.37 ± 10.82 μM; 9 replicates) and strain T2758 (mean IC_{50} of 32.37 ± 7.15 μM; 3 replicates), which contains the UL97 mutation T409M observed in a MBV-treated subject (Strasfeld et al., 2010).

Finally, the possibility of a synergistic effect by ART-MBV combination treatment was experimentally addressed. Previous studies have shown that wild-type HCMV strains have IC_{50} values for MBV close to 0.1 μM when determined in HEL fibroblasts, but are ~100-fold higher (10 μM range) when tested in HFF cultures, presumably reflecting metabolic differences in the two cell types (Chou et al., 2006). When tested in a similar manner, the presence of ART at a fixed concentration of 2 μM (less than its own IC_{50} value) reduced the measured MBV IC_{50} for wild type control strain T2211 in HFF cultures, from 12.1 ± 2.5 to 0.20 ± 0.12 μM (8 replicates each, performed simultaneously over 4 setup dates). Thus, the fractional inhibitory concentration (ratio of IC_{50} values of the combination versus MBV alone) is very low at 0.02., comparable to previously reported MBV-drug combination treatments in HCMV-infected HFF cultures (Chou et al., 2006).

In conclusion, this report describes novel characteristics of the strong anti-cytomegaloviral activity of ART. The main findings are: (i) ART, but not ART-related sesquiterpene compounds, possesses strong anti-cytomegaloviral activity targeted to very early events of viral replication, (ii) ART treatment of HCMV-infected cells immediately post-virus adsorption exerts antiviral activity, while an exclusive preincubation of cells prior infection is inefficient, (iii) repeated, additive post-infection incubation of ART provides optimal efficacy *in vitro*, (iv) ART is similarly active against therapy-resistant HCMV mutants and (v) ART shows a pronounced synergistic effect in combination with MBV. An important finding, possibly influencing future clinical applications of ART, was seen in the increase of antiviral efficacy under conditions when ART was applied consecutively post-infection in fractional doses rather than a single dose. This finding refers to the short half-life of the drug, i.e. $t1/2$ <1 h in human cells (Efferth et al., 2004, 2008). Although the antiviral mode of action of ART is not yet explained in detail, it appears very probable that ART acts through a unique mechanism not known for other herpesviral drugs. ART interferes with a very early stage of viral replication and inhibits the synthesis of viral immediate early proteins, most probably as a result of the inhibition of virus-supportive cellular activation pathways (Efferth et al., 2002, 2008). Here, the activity of ART was demonstrated against a wider range of defined drug-resistant HCMV mutants than previously reported, confirming that this host cell-directed antiviral mechanism is independent from viral strain variation. This increases the likelihood that ART may have a therapeutic role in the treatment of infections that have become resistant to standard therapy. Of particular interest is the synergism between ART and the developmental drug MBV identified in this study. Because of clinical reports of an antiviral effect of MBV (Avery et al., 2010; Strasfeld et al., 2010) in cases of HCMV infection resistant to conventional treatment, optimizing the potency and use of this drug remains a worthwhile goal in conjunction with planned clinical trials. The synergism with ART appears to have great clinical prospect because of the potential use in a wide range of patients requiring anti-cytomegaloviral treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.07.018.

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