Anti-cytomegalovirus applications of the intrinsically active drug carrier lactoferrin
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Synergy of bovine Lactoferrin with the Anti-Cytomegalovirus drug Cidofovir in vitro.

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Submitted
**ABSTRACT**

Human cytomegalovirus (HCMV) causes severe morbidity and mortality in immunocompromised patients. Treatment of HCMV infections with conventional antiviral drugs like ganciclovir and cidofovir has major drawbacks. Serious side effects and resistance to antiviral therapy are well-recognised phenomena. Therefore, combination therapies using drugs with different antiviral mechanisms are considered.

Synergy between Lactoferrin (LF), an antibacterial, antimycotic and antiviral protein, and the antiviral drugs acyclovir, ganciclovir, foscarnet and cidofovir was measured, using an *in vitro* test system with the recombinant RC256 HCMV strain. Antiviral activity of separate antivirals and synergy/antagonism of combinations of LF and antivirals was calculated using standard methods.

Combination of LF with acyclovir and also foscarnet resulted in antagonism in CMV inhibition up to 55%. Antagonism of acyclovir with LF was not due to significant binding of acyclovir to LF. When LF and ganciclovir were combined, neither synergy nor antagonism was observed. Strikingly, the combination of LF and cidofovir resulted in synergy up to 42% in the low concentration range of both antivirals. An improved penetration of cidofovir by membrane perturbation of lactoferrin was experimentally excluded. Rather, the synergistic effect was explained by inhibition of two subsequent steps in the viral replication: penetration of the target cell by HCMV and intracellular synthesis of HCMV DNA.

We conclude that LF might be a potential candidate for combination therapy with cidofovir and may also be used for cell selective delivery of cidofovir to target cells.
INTRODUCTION

Human Cytomegalovirus (HCMV) is a member of the β-herpes virus family. Like other herpes viruses, it causes a persistent and latent infection, which can reactivate under circumstances of immunosuppression \(32,45,46\). In healthy subjects, primary infection with HCMV generally occurs without any symptoms and therefore passes mostly unnoticed. However, in individuals lacking a proper immune response like (premature) neonates and organ transplant or AIDS patients, primary infections or reactivations can cause severe morbidity or mortality.

Infections, or reactivations, with HCMV are among the most frequently occurring opportunistic infections in the late stage of AIDS \(27,35\). In these patients, HCMV disease may result in a systemic end-organ disease throughout the body such as the gastro-intestinal tract, lungs, liver, retina and the central nervous system \(5,12,40\). Moreover, HIV-infected patients who are seropositive for HCMV, appear to progress more rapidly to AIDS \(13,14,38,44\). In HIV-infected patients the occurrence of persistent CMV infections with high CMV load is associated with a decreased survival time \(5,8,40\).

Active HCMV infections are commonly treated with drugs like ganciclovir, foscarnet and, more recently, with cidofovir (Vistide, HPMPC). These drugs all exhibit a potent antiviral activity in vitro and in vivo. However, treatment with these conventional antiviral drugs is hampered by a number of limitations. Firstly, long-term treatment with these drugs will lead to undesirable side effects like nausea, diarrhoea, bone marrow suppression and nephrotoxicity; complicating treatment of the infection \(1,7,31,34,47\). Secondly, due to the prolonged antiviral therapy, resistance to antiviral therapy arises almost invariably, leading to relapses of disease \(8,19,40\). Therefore, the development of drug resistance is an important complication in the long-term treatment of HCMV infections in immunosuppressed patients.

Combination therapy will reduce the incidence of drug resistance. Using antiviral compounds that operate through different antiviral mechanisms will lead to a simultaneous inhibition at different steps of the viral replication cycle. Another approach is the application of specific glycoproteins as intrinsically active carrier molecules to specifically deliver antiviral drugs to infected cells, in which
the drug will interfere with virus replication\textsuperscript{29}. These intrinsically active drug carriers contribute to the therapeutic effect, apart from delivering a coupled drug the required cell type.

Lactoferrin (LF) is an 80 kD glycoprotein that could be used as an intrinsically active carrier protein.\textit{In vivo}, LF is present in the secondary vesicles of neutrophilic granulocytes\textsuperscript{4,24}. In addition, LF is produced by the mammary glands during lactation and also by mucosal epithelial cells at the mucosa\textsuperscript{2,18,25}. Therefore, LF is present in many body fluids such as tears, saliva, seminal and vaginal fluids and breast milk\textsuperscript{23,25}.

At these sites LF is a major constituent of the non-specific immune defence system against microbial infections. LF has bacteriostatic and bactericidal effects against Gram negative and Gram positive bacteria\textsuperscript{18,25}. Antifungal effects, in particular against\textit{Candida} species, have also been described\textsuperscript{21,23,25}. Finally, LF also exerts antiviral activity against a wide range of viruses including HSV-1, HSV-2, rotavirus, RSV, HIV and HCMV\textsuperscript{15,16,26,37,41}. Plasma LF concentrations are significantly decreased in endstage AIDS patients, rendering these patients more sensitive to opportunistic infections\textsuperscript{43}, especially since the specific immune system of these patients is severely disturbed.

As we have shown, that LF acts synergistic with antifungal drugs against several clinical isolates of\textit{Candida} species\textsuperscript{21}, we hypothesise that LF also synergises with antiviral drugs. In this study we aimed to investigate the potential synergistic antiviral activity of LF with the conventional antiviral drugs acyclovir, ganciclovir, foscarnet and cidofovir.

\textbf{MATERIALS \& METHODS.}

\textit{Preparation of virus stocks}.

Human Foetal Lung Fibroblasts (FLF), between passage 7 and 18, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Biowhittaker Europe, Verviers, Belgium), supplemented with 10\% foetal calf serum (Brunschwig Chemie, The Netherlands), 200mM L-glutamin (Gibco BRL, Paisley Scotland), 60 $\mu$g/ml gentamycine sulphate (Gibco BRL, Paisley Scotland) and 25 $\mu$g/ml
Amphotericin B (Fungizone®, Bristol Myers Squibb Company, Woerden, The Netherlands) at 37°C, 100% humidity and 5% CO₂.

Infective virus was prepared by adding RC256 virus (kindly provided by E. Mocarski) at a Multiplicity of Infection (MOI) of 0.1 to subconfluently growing FLF in large tissue culture flasks (162 cm², Corning Costar, Cambridge, UK). Infection was allowed to proceed until maximal cytopathic effect was achieved. Usually this was achieved approximately 10 days post infection. Cells were detached using a cell scraper and centrifuged subsequently. Supernatant was aliquotted, frozen in liquid nitrogen and stored at -80°C.

**Antiviral compounds.**

Bovine LF (Numico Research B.V., Wageningen, The Netherlands) was dissolved in culture medium, supplemented as described above but with 3% foetal calf serum, at a concentration of 3.0 mg . ml⁻¹. Conventional antiviral drugs used in these experiments were acyclovir (Genthon B.V., Nijmegen, The Netherlands), ganciclovir (Cymevene®, Roche Nederland B.V., Mijdrecht, The Netherlands), foscarnet (Foscavir®, Astra Pharmaceuticals, Zoetermeer, The Netherlands) and cidofovir (Vistide©, Pharmacia & Upjohn S.A., Luxembourg). All drugs were reconstituted according to manufacturer’s advice and subsequently diluted to a concentration of 3.0 mg . ml⁻¹ in culture medium, supplemented with 3% foetal calf serum.

**Virus assay.**

Antiviral activity of compounds was tested as described by Hippenmeyer et al. with minor modifications. One day prior to the assay, FLF were seeded into 96-wells plates (Corning Costar, Cambridge, UK) at a density of approximately 10,000 cells per well. The next day, medium was refreshed with culture medium, supplemented with 3% foetal calf serum.

In order to test the activity of a single compound used, two-fold serial dilutions of a compound were added to the wells on each plate. Combinations of LF with one of the conventional antivirals were added in a 8x8 two-fold dilution matrix. Cells were preincubated with the antivirals for 15 min. at 37°C, 100% humidity and 5% CO₂, prior to addition of virus. Preincubation of cells is essential for the antiviral activity of LF.
Subsequently, RC256\textsuperscript{39} was added at an MOI of 1. As a negative control, a series of wells was left uninfected with RC256, nor incubated with antivirals. As a positive control, cells were infected with RC256 and not treated with any antiviral drug or LF.

Plates were incubated at 37°C, 100% humidity and 5% CO\textsubscript{2} for three days. Subsequently, medium was replaced with 200 µl of 4.0 mg . ml\textsuperscript{-1} ONPG (2-Nitrophenyl-ß-D-Galactopyranoside, Boehringer Mannheim, Germany) in 80 mM Na\textsubscript{2}HPO\textsubscript{4}, 20 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.1 mM MgCl\textsubscript{2}, 2.0 mM MgSO\textsubscript{4}, 40 mM ß-Mercapto-ethanol, 0.1% Triton X-100, final pH 7.4. After 2 hrs incubation at 37°C, the staining reaction was stopped using 1.0 M Na\textsubscript{2}CO\textsubscript{3}. Extinctions were measured at 405nm, using Softmax Pro software. Experiments were independently repeated for at least three times.

**Calculation of Synergy.**

The results obtained with the single antiviral compounds against RC256 were used to calculate the antiviral effect. Maximal infection, achieved in the positive controls was set at 100%. Extinctions of the negative controls were set at 0%. To analyse the interactions of LF with the other conventional antiviral compounds, a three-dimensional analytical method described by Prichard\textsuperscript{21,33} was used. In brief, dose-response curves of the individual drugs were used to calculate theoretical additive interactions. Subsequently, these interactions were subtracted from the experimental interactions. This subtraction was performed to detect interactions that are higher than expected on the combined antiviral activity of the single compounds, which is defined as synergy.

If interactions were solely additive, this would appear as a horizontal surface at 0% virus inhibition above the calculated inhibition surface. In case of synergy, peaks above this 0% surface will be found. Antagonistic activities would result in values under this 0% surface. For example, a calculated peak of +40% would indicate a synergistic antiviral effect of 40%, meaning that at this combination of drug concentrations viral replication is 40% more efficiently inhibited than one would expect based on the activity of either drug alone. Similarly, a peak of –50% would mean that viral replication is inhibited 50% less efficiently as expected on individual dose-response curves of the antivirals, indicating antagonism.
Binding assays.

Ultrafiltration experiments were performed to study protein binding of either acyclovir and lactoferrin, or Cidofovir and lactoferrin. Serial ten-fold dilutions of lactoferrin (ranging from 78 to 0.078 µg . ml.⁻¹) were added to a constant amount (0.75 MBq) of either ³H-Acyclovir (Campro Scientific, Veenendaal, The Netherlands) or ³H-Cidofovir (Campro Scientific, Veenendaal, The Netherlands). These mixtures, in a total amount of 1 ml, were added to filter tubes (Vivaspin Concentrators, Vivascience, Binbrook, UK). The tubes were centrifugated at 5,000g for 1 min, producing maximally 0.1 ml of ultrafiltrate. Subsequently, the amount of radioactivity of both the lactoferrin solution and the protein-free filtrate was counted using Ultima Gold XR (Packard, USA) in a Beckman counter. The amount of protein binding was expressed as the unbound fraction of ³H-labeled drug. The fraction of unbound drug (fu) was calculated as the drug concentration in the protein solution (Cp), divided by the farmacon concentration in the filtrate (Cf). Therefore fu = Cf / Cp. If no protein binding is occurring, Cf equals Cp, hence fu = 1.

Cellular accumulation studies in FLF.

Absorption studies with ³H-labeled Cidofovir were performed to investigate a possible increase in uptake of Cidofovir in FLF, caused by addition of LF to the cells. In brief, FLF were seeded in 24-wells plates and were maintained in culture medium as described earlier. When the cells had reached confluency, dilution series of LF (0.039 - 390 µg . ml⁻¹) and a constant amount of ³H-labeled Cidofovir (0.75 MBq, Campro Scientific) were added to each well. Cells were maintained at 37°C, 5% CO₂ and 100% relative humidity for three days with, or without presence of the HCMV RC256-strain (MOI = 0.1). At three days post infection, the cells were washed with PBS and lysed with 1M NaOH. The lysate was dissolved in Ultima Gold XR (Packard, USA) and the amount of radioactivity was counted.
RESULTS

Inhibition of RC256.

The antiviral activity of each individual compound against HCMV RC256 was determined. The IC\textsubscript{50}-values of the compounds are shown in Table 1. The inhibitory concentrations of the conventional anti-CMV drugs were comparable to those described in literature \cite{1,7}, although the IC\textsubscript{50}-value of LF is somewhat lower than described earlier, using the laboratory strain AD169 \cite{16}. This is probably due to the different assay system we used. Furthermore, RC256 is a recombinant derivative of the Towne strain, whereas AD169 was used earlier. Table 1 also shows a relatively high IC\textsubscript{50}-value for acyclovir, confirming that acyclovir alone does not have a significant antiviral activity against HCMV.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (µg \cdot ml\textsuperscript{-1})</th>
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<tbody>
<tr>
<td>Acyclovir</td>
<td>&gt;125</td>
</tr>
<tr>
<td>Ganciclovir</td>
<td>9.0 ± 1.2</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>48.0 ± 1.1</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>7.0 ± 1.0</td>
</tr>
</tbody>
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*Table 1: Antiviral activities of bovine Lactoferrin and conventional antiviral drugs against RC256 HCMV-strain. IC\textsubscript{50} -values were calculated using a 4-parameter curve fitting algorithm (Graphpad Prism Software).*

Antagonistic activities of acyclovir with LF.

Combination of acyclovir with LF resulted in a clear antagonistic effect that amounted to maximally 55%. This implies that LF is 55% less active against HCMV as anticipated on basis of the respective dose-response curves (Fig.1). The maximum amount of antagonism was observed at concentrations of about 0.8 µg \cdot ml\textsuperscript{-1} both for LF and acyclovir.
Antagonistic activities between LF and Acyclovir. The maximum amount of antagonism reached up to 53.7% (±9.2% SEM). Values are means of 3 independent experiments.

Combination of ganciclovir with LF.
Overall, the combination of LF with ganciclovir did not show distinct synergy, nor antagonism in the concentration ranges that were studied (Fig. 2). Maximally, 10% synergy was observed in concentrations of about 8 μg . ml⁻¹ both for LF and ganciclovir. Maximally, 10% antagonism was observed for 4.0 μg . ml⁻¹ LF and 2.0 μg . ml⁻¹ ganciclovir.

Combination of cidofovir with LF.
Combination of LF with cidofovir resulted in a significant synergistic effect (Fig. 3). The maximum amount of synergy observed was 42%, at a concentration of approximately 8 μg . ml⁻¹ LF and 4 μg . ml⁻¹ of cidofovir. In other words, HCMV was inhibited 42% more efficiently than theoretically expected on basis of the individual dose-response curves of both compounds.
Fig. 2: Interaction of Lactoferrin with ganciclovir. Neither antagonism nor synergy was observed. Values are means of 3 independent experiments.

Fig. 3: Synergy between Lactoferrin and Cidofovir. Maximum amount of observed synergy reached up to 42% (±10.6% SEM). Values are means of 5 independent experiments.
Combination of foscarnet with LF.

Combination of LF with foscarnet resulted in clear antagonistic effects (Fig. 4). The maximum amount of antagonism was 51% at a concentration of 8 µg . ml⁻¹ and 2 µg . ml⁻¹ for LF and foscarnet respectively.

![Fig. 4: Antagonistic activities between LF and Foscarnet. Antagonism was maximally 51% (± 11.2 SEM). Values are means of 5 independent experiments.](image)

Binding of acyclovir and cidofovir to LF.

The observed antagonism of LF with ACV could be explained by the binding of ACV to LF, rendering LF antivirally inactive. However, no significant binding of ³H-Acyclovir bovine LF could be detected by ultrafiltration studies (data not shown). Similar results were obtained with ³H-Cidofovir. For both ³H-Acyclovir and ³H-Cidofovir the fu exceeded 0.95.

Cellular accumulation studies in FLF.

The observed synergistic effects between LF and Cidofovir could be a result of an increased uptake of Cidofovir due to the presence of LF. However, addition of different concentrations of LF to the culture medium did not result in an enhanced uptake of Cidofovir by FLF. This was observed both in infected as in non-infected FLF (data not shown).
DISCUSSION

Chronic or prophylactic treatment is needed to prevent rapid progression of AIDS and fatal HCMV disease in AIDS patients. However, chronic treatment is often complicated by side effects and the development of viral resistance to drug therapy. The use of combination therapy, in which drugs with distinct antiviral mechanisms are used simultaneously, may reduce the risk of development of antiviral drug resistance. In this respect, it is essential that the combined drugs should not antagonise each other, but should preferably be synergistic. This consideration also applies to the use of intrinsically active protein carriers that can deliver covalently coupled antiviral agents to their target cells, but also contribute to the antiviral effect themselves.

In this study, we describe the effects of combinations of the antimicrobial protein LF with some conventional anti-cytomegalovirus drugs. Our studies show that combination of LF with ACV, or foscarnet, leads to clear antagonistic effects, while combination of LF with GCV did not result in consistent synergy or antagonism. In contrast, combination of LF with Cidofovir led to significant synergistic effects.

The prodrug acyclovir (ACV, Fig. 5a), a guanosine analogue, is frequently used as treatment against HSV-1, HSV-2 and Varicella zoster infections. Acyclovir requires phosphorylation to become antivirally active. Acyclovir enters the cell by passive diffusion and is selectively phosphorylated by a viral encoded thymidine kinase (tk). The monophosphorylated ACV (ACVp) is phosphorylated by host cell encoded kinases to the triphosphorylated ACV (ACVppp) which is the antivirally active form. ACVppp inhibits viral DNA polymerase by competing with 2'-deoxy guanoside triphosphate as a substrate and prevents DNA chain elongation (reviewed by Balfour and Whitley). Resistance to acyclovir is mediated by a mutation in the viral tk-gene, or viral DNA polymerase. Cytomegalovirus does not encode a thymidine kinase, thus CMV is not sensitive for treatment with ACV.
In contrast, ganciclovir (GCV), a guanosine analogue that closely resembles the structure of ACV (Fig. 5b), is a potent inhibitor of CMV replication. Similar to ACV, GCV requires phosphorylation to ganciclovir triphosphate (GCVppp) to exert its antiviral activity. In HCMV-infected cells, this phosphorylation of GCV is mediated by UL97, a phosphotransferase encoded by CMV. In addition, GCV can also be phosphorylated by tk, encoded by several herpes viruses. GCVppp competes with 2'-deoxyguanosine as a substrate for the viral DNA-polymerase. Unlike ACVp, the GCV monophosphate (GCVp) is built into the DNA-chain, but is not able to prevent DNA chain elongation due to it’s 3’-OH-group, which permits primer-template extension \(^1,7\). Mutations in tk or UL97, or mutations in viral DNA polymerase (UL54) mediate resistance to GCV.

Cidofovir (Fig. 5c), a cytosine analogue, enters the cell by fluid-phase endocytosis \(^31,34\). Unlike ACV and GCV, Cidofovir poorly crosses the cell membrane due to the negatively charged phosphonate moiety. Yet a major advantage of this drug is the fact that it does not need a prior activation by virally encoded kinases. Therefore, mutations in TK or UL97 do not confer viral resistance. After specialised, carrier mediated uptake by a transporter protein in the cell, Cidofovir is phosphorylated by cellular kinases to Cidofovir diphosphate, which is the active form of the drug. Cidofovir diphosphate selectively inhibits viral DNA-polymerase by competition with 2’-deoxycytosine triphosphate. Like

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Fig. 5a: Chemical structure of acyclovir (ACV).

Fig. 5b: Chemical structure of ganciclovir (GCV).
GCVp, incorporation of Cidofovir diphosphate does not lead to an immediate chain termination. Only after incorporation of two subsequent Cidofovir diphosphate molecules, DNA replication comes to a full stop (reviewed by Safrin and Naesens) \(^{31,34}\). Resistance to Cidofovir has already been reported and is mediated by mutations in the viral DNA-polymerase \(^{31,34}\).

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**Fig. 5c: Chemical structure of Cidofovir (HPMPC).**

Foscarnet (Fig. 5d), trisodium phosphonoformate is an inorganic analogue of inorganic pyrophosphate \(^{31}\). Foscarnet is used as treatment of HCMV-infections that are resistant to GCV therapy. The antiviral mechanism of foscarnet is the complex formation with viral DNA polymerase at its pyrophosphate-binding site. Therefore, cleavage of pyrophosphate from nucleoside pyrophosphates, thus primer-template extension, is blocked \(^{31}\).

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**Fig. 5d: Chemical structure of Foscarnet.**

LF probably interferes with the viral entry into the host cell, since preincubation of target cells with LF is essential for the antiviral effect. When LF is added at increasing times after preincubation of target cells with HCMV, the protective effect gradually decreases \(^{16}\). It has been shown previously that LF binds to heparan sulphate proteoglycans (HSPG) \(^{42}\), which are also used by herpes viruses as a primary anchor to infect the target cells \(^{6,36}\). Thus LF accomplishes at least part of the antiviral effect by preventing virus from docking onto a target cell.
Regarding the synergistic activities between LF and Cidofovir as reported in this study, the mechanism leading to synergy between these molecules remains to be established. It could be hypothesised that uptake of Cidofovir by the target cell is increased by LF. This could be due to the positively charged N-terminal stretch of LF, which is responsible for binding to the negatively charged HSPGs 42, but can also be responsible for binding the negatively charged moiety on Cidofovir. However, the binding experiments we performed did not reveal any binding of LF to Cidofovir.

Another mechanism for the enhanced uptake of Cidofovir could be a result of membrane perturbation by LF, resulting in increased fluid-phase endocytosis and cellular uptake of Cidofovir. Concomitant uptake of LF with cidofovir could render the combination of drugs more effective against HCMV as theoretically expected on basis of individual dose-response curves. However, we were not able to detect an increased uptake of Cidofovir due to the presence of LF in the culture medium. Therefore, it is most likely that the synergistic effect of Cidofovir and LF lies in the fact that both drugs interfere at different stages of viral replication. LF interferes with entry of the virus, leading to a lower viral load in the target cells. Subsequently, Cidofovir may inhibit this lower amount of virus more efficiently. However, in the case of Ganciclovir such a synergistic effect was not observed and this may either be due to the principle difference in phosphorylation steps of the two antiviral compounds, or to the more polar character of Cidofovir.

The antagonistic effect between LF and the studied antiviral drugs are not clear. Although the structures of ACV and GCV are highly similar, combination of LF with ACV resulted in antagonism, whereas the combination GCV with LF did not show either antagonistic or synergistic effects. Since ACV itself has no antiviral activity against HCMV in the concentration ranges we used, the observed antagonism may only imply that ACV impaired the antiviral effect of LF. This impairment of the antiviral effect of LF cannot be explained by direct binding of ACV to LF, since the binding studies we performed did not reveal any binding of neither ACV nor Cidofovir to LF in vitro.

We conclude that combination therapy consisting of LF with Ganciclovir and in particular Cidofovir might be a promising treatment for HCMV infections in immunocompromised patients and deserves further investigation. We are
presently studying whether covalent binding of Cidofovir yields an effective drug targeting preparation for CMV-infected target cells. Such an approach was taken by us \(^3,^{20,22,28,30}\) and others \(^{9,11}\) for HIV or Hepatitis B-virus targeting using lactosaminated proteins and polymer carriers.

**Acknowledgements**

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REFERENCES


