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Inhibition of human cytomegalovirus signaling and replication by the immunosuppressant FK778

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Abstract

FK778 (Fujisawa Healthcare Inc.) is an immunosuppressant structurally similar to A771726, the active metabolite of leflunomide (Aventis Pharmaceuticals), but with a clinically relevant shorter serum half-life. Leflunomide, a tolerated and efficacious immunosuppressive agent in patients receiving allograft transplantations, was reported to be active against HCMV and HSV-1. Here we report that FK778 is a potent and effective inhibitor of HCMV, and that its mode of antiviral action appears to mirror the biochemical mechanisms elsewhere described to be responsible for its immunosuppressive properties: inhibition of protein tyrosine phosphorylation and inhibition of cellular de novo pyrimidine biosynthesis. Initial HCMV-mediated activation of the EGF receptor/phosphatidylinositol 3-kinase (PI3-K) pathways and Sp1 and NF-κB were partially inhibited by FK778. The second tier (phase) of PI3-K, Sp1, and NF-κB induction by HCMV was more sensitive to FK778. Treatment of HCMV-infected cells with FK778 prevented the appearance of HCMV proteins some 12–24 h post infection, and inhibited viral DNA synthesis. In our assays, leflunomide also reduced HCMV DNA levels. The antiviral activity of FK778 was reversed in cell culture by treatment with uridine, consistent with specific inhibition of dihydroorotate dehydrogenase (DHODH), a required enzyme in the de novo biosynthesis of pyrimidines. This report substantiates the clinical possibility of a single drug treatment to achieve immunosuppression and inhibit opportunistic herpesvirus infections. Our results differ from descriptions of leflunomide acting as an inhibitor of HCMV cytoplasmic capsid formation. Additionally, this study indicates that DHODH may be an effective cellular antiviral target.

Keywords: Arava; Leflunomide; A771726; DHODH; EGF receptor

1. Introduction

Human cytomegalovirus (HCMV) is a β-herpesvirus with high seroprevalence but most infected individuals are asymptomatic (Landolfo et al., 2003). However, HCMV is responsible for significant morbidity and mortality in immunocompromised populations. This virus is a leading cause of birth defects, often causing mental retardation and deafness (Plotkin, 1999). HCMV is a common opportunistic pathogen in transplant recipients and is associated with allograft rejection (Rawlinson and Scott, 2003). Acquired immune deficiency syndrome (AIDS) patients frequently lose their sight due to HCMV retinitis (Scholz et al., 2003). There is evidence that HCMV may play a role in the etiologies of atherosclerosis, coronary artery restenosis, and inflammatory bowel diseases (Rawlinson and Scott, 2003).

The most widely used clinical therapies for the prophylaxis and treatment of HCMV infections are the small molecule inhibitors: pyrophosphate analog foscarnet and nucleoside analogs cidofovir and ganciclovir (and its valine ester pro-drug valganciclovir) (Griffiths, 2002). These virostatic drugs block viral DNA polymerase but do not prevent HCMV from entering host cells or inducing multiple signal transduction events, nor do they halt virus production of

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immediate-early and early viral proteins (Huang, 1975b; Mar et al., 1983, 1985). Additional limitations include the clinical prevalence of drug-resistant and cross-resistant strains of HCMV (Erice, 1999), as well as dose limiting renal (foscarnet and cidofovir) and bone marrow (ganciclovir) toxicities (Griffiths, 2002). Other antiviral agents employed are the intraocularly injected antisense phosphorothioate oligonucleotide fomivirsen (Perry and Barman Balfour, 1999) and HCMV hyperimmune globulin (Snydman, 2001). The mechanisms of action for HCMV hyperimmune globulin and fomivirsen have not been defined, but they appear to act at or around the entry and entry/immediate-early stages of viral replication, respectively (Andreoni et al., 2002; Azad et al., 1993).

Leflunomide (Fig. 1; Arava, Aventis Pharmaceuticals) is a marketed therapeutic agent for the treatment of rheumatoid arthritis and autoimmune disorders (Sanders and Harisdangkul, 2002). It is a tolerated and efficacious immunosuppressive agent in patients receiving allograft transplantations (Williams et al., 2002). Reports that leflunomide was also active against HCMV (Waldman et al., 1999a,b) and herpes simplex virus type 1 (HSV-1) (Knight et al., 2001) indicated the clinical possibility of a single drug treatment to achieve immunosuppression and inhibit opportunistic herpesvirus infections. An immunosuppressive drug with activity against HCMV would have a significant advantage over currently marketed therapeutics. The immunosuppressive activity of A771726 (Fig. 1), the active metabolite of leflunomide, has been described to result from the inhibition of dihydroorotate dehydrogenase (DHODH), a required enzyme in the de novo biosynthesis of pyrimidines (Davis et al., 1996). Consistent with a mode of immunosuppressive action involving inhibition of de novo pyrimidine biosynthesis, in some cases the effects of leflunomide have been reversed in vitro by the addition of uridine (Williamson et al., 1995; Chong et al., 1999), presumably supplying cells with sufficient pyrimidines via salvage pathways. It has also been proposed that leflunomide's immunosuppressive properties are due to its inhibition of protein tyrosine kinase phosphorylation (Xu et al., 1995; Mattar et al., 1993).

Initial events in the HCMV replication cycle involve signal transduction via phosphorylation of the epidermal growth factor (EGF) receptor (Wang et al., 2003) and activation of phosphatidylinositol 3-kinase (PI3-K) (Johnson et al., 2001). Cellular kinases Akt and p70S6K were activated in a PI3-K-dependent biphasic manner following HCMV infection (Johnson et al., 2001). HCMV infection also resulted in biphasic induction of the cellular transcription factors NF-κB and Sp1 (Yurochko et al., 1995, 1997a,b). EGF phosphorylation and the first phase of PI3-K, NF-κB, and Sp1 induction are consistent with virion receptor/ligand mediated signaling event(s) in the absence of de novo protein synthesis (Wang et al., 2003; Yurochko et al., 1995, 1997a,b). The second phase of PI3-K, NF-κB, and Sp1 induction required HCMV (and cellular) protein expression, and was detectable from approximately 4–12 hpi, remaining elevated relative to mock-infected cells for the remainder of infection (Yurochko et al., 1995, 1997a,b; Johnson et al., 1999).

Since leflunomide demonstrated inhibition of tumor necrosis factor (TNF)-stimulated NF-κB induction (Manna et al., 2000), and blocked phosphorylation of the EGF receptor (Mattar et al., 1993), it might be expected to affect the first, second, or both tiers of HCMV-mediated PI3-K signaling and NF-κB activation. Similarly, deprivation of cellular pyrimidine building blocks by leflunomide ought to affect the replication of herpesvirus DNA. Surprisingly, Waldman et al. (1999a,b) reported that leflunomide acted late in the replication cycles of HCMV and HSV-1, without affecting viral protein synthesis or viral DNA synthesis, instead inhibiting cytoplasmic capsid formation (Knight et al., 2001).

FK778 (Fig. 1) is an orally bioavailable synthetic analog of A771726 currently being developed as an immunosuppressive/anti-inflammatory therapeutic by Fujisawa Healthcare Inc. (Jin et al., 2002). Our goals in this study were to examine the possibility that FK778 might possess antiviral activity against HCMV, and to determine the molecular basis of that viral inhibition. Previous structure–activity relationship studies (Williamson et al., 1995; Kuo et al., 1996) along with the structural similarity between A771726 and FK778 (Fig. 1) might predict that these compounds exhibit similar immunosuppressive and antiviral activities. For example, the immunosuppressive properties of HR325, a synthetic analog of A771726, were reversible upon treatment with exogenous uridine (Thomson et al., 2002). Also, antiviral activity against LP-BM5 virus-induced disease has been reported for a structurally unrelated inhibitor of DHODH (Scott et al., 1993). Here we report that FK778 is a potent and effective inhibitor of HCMV, and that its mode of antiviral action appears to mirror the biochemical mechanisms elsewhere described to

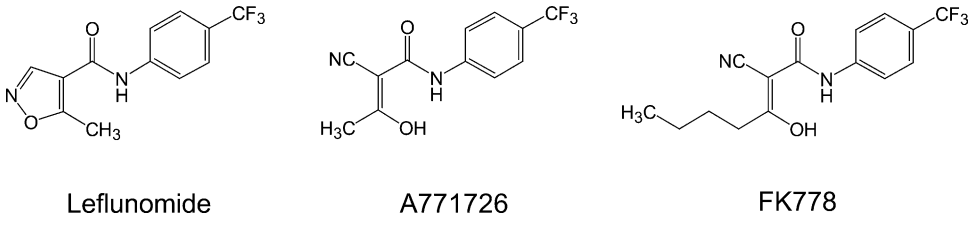

Fig. 1. The chemical structures of leflunomide ("Arava," Aventis), A771726, and FK778 (Fujisawa Healthcare Inc.).

be responsible for its immunosuppressive properties: inhibition of protein tyrosine phosphorylation and inhibition of cellular de novo pyrimidine synthesis.

2. Materials and methods

2.1. Antiviral compounds, reagents, and antibodies

FK778 (2-cyano-3-oxo-*N*-[4-(trifluoromethyl)phenyl]-6-heptanoic acid amide; previously known as HMR 1715 and MNA 715) was supplied by Fujisawa Healthcare Inc. (Osaka, Japan) and ganciclovir (9-[{1,3-dihydroxy-2-propoxy} methyl]guanine; GCV) was purchased from the University of North Carolina Chapel Hill Hospital pharmacy. Leflunomide (5-methylisoxazole-4-[4-trifluoromethylcarboxanilide]; Arava) was purchased from Sigma (St. Louis, MO). Compounds were prepared as 10–20 mg/mL stocks in dimethylsulfoxide (DMSO) and stored at −20 °C. Cytogam (HCMV hyper immune globulin) was obtained from MedImmune (Gaithersburg, MD). Uridine was purchased from Sigma.

Primary monoclonal antibodies specific for HCMV IE1, IE2, UL84, and UL94 proteins were prepared in our laboratories as described previously (He et al., 1992; Kowalik et al., 1994; Wing et al., 1996). Antibody (Ab) to gB (13-127-100) was purchased from Advanced Biotechnologies (Columbia, MD). Ab to β-actin (CP01) and its corresponding horse radish peroxidase (HRP)-conjugated secondary Ab were purchased from Oncogene (San Diego, CA). Rabbit Ab to EGF receptor was a gift of Dr. H. Shelton Earp III, while mouse Ab to EGF receptor (SC528) was purchased from Santa Cruz (Santa Cruz, CA). HRP-conjugated Ab to phosphotyrosine (RC20H) was purchased from Transduction Laboratories (Lexington, KY). Ab to Akt (9272) and p70S6K (9202), and Ab specific for Akt phosphorylated at Ser 473 (9275) and p70S6K phosphorylated at Thr 389 (9205) were purchased from Cell Signaling (Beverly, MA). HRP-conjugated Ab to mouse (A9044) and rabbit (A6154) IgG were purchased from Sigma; secondary Ab to human IgG (401455) was purchased from Calbiochem (La Jolla, CA).

2.2. Cell culture and viral infection

Primary human embryonic lung fibroblasts (HEL 299) were purchased from the American Tissue Culture Collection (Manassas, VA) and were cultured in minimum essential media (MEM) supplemented with 10% fetal bovine serum as described previously (Johnson et al., 2001). The Towne strain of HCMV (passages 36–42) was propagated as described previously (Huang et al., 1973). HCMV clinical isolates Major and TW-087 were obtained from human prostate and from the cervix of a pregnant woman, respectively, and have been described (Huang et al., 1976). To investigate the effect of FK778 on virus-induced signaling, cells were grown to confluence, then serum starved for 48 h in MEM. Cells were infected with HCMV at a multiplicity of infection (MOI) of 2–5 plaque-forming units (PFU)/cell that was purified through a sucrose cushion to eliminate cytokines and growth factor contamination.

2.3. Plaque and titer reduction assays

To determine the effects of FK778 upon viral pathology, confluent HEL fibroblasts in 24-well plates were incubated at 37 °C in an atmosphere of 6% CO_2 for 1 h in the presence or absence of various concentrations of FK778 in Dulbecco's modified Eagle medium (DMEM). Cells were then infected with HCMV (Towne strain) at approximately 400 PFU per well. Following a 1 h adsorption, the inoculant was removed, and a 1% methylcellulose (methocell) overlay containing DMEM with heat-inactivated 4% FBS in the presence or absence of various concentrations of FK778 was added. Seven days post infection, cell monolayers were stained with crystal violet, and plaques were scored by inverted light microscopy (Wentworth and French, 1970). Data are presented as the percentage of plaques in the wells lacking drug (positive controls). In these experiments, some 170–200 plaques were present in each positive control well.

To determine the effects of FK778 upon the release of viral progeny from infected cells, pre-treatment and infection of HEL fibroblasts in the presence and absence of FK778 (32 μM) and GCV (70 μM) were performed as described above. Following virus adsorption, cultures were nourished in medium lacking methocell. At six days post infection the extracellular media was collected and serial dilutions were used to inoculate HEL monolayers (Prichard et al., 1990). One hour later, innoculum was removed, fresh media containing 1% methocell and 6% FBS was added, and the cultures were incubated and scored as described above for plaque reduction assays. Since results were plotted on a log scale, wells with zero plaques in the lowest dilution were assigned a titer of 1 PFU/mL.

2.4. Cytotoxicity measurements

To quantify the cellular cytotoxicity of FK778 in static monolayers of HEL cells, cultures were prepared under the conditions described for plaque reduction assays, except that virus innoculum and methocell were omitted. Seventy-two hours post incubation with selected concentrations of FK778, the media was removed and cell viability was assessed with a tetrazolium colorimetric dye assay according to the manufacturer's instructions (Promega, Madison, WI). At each concentration of FK778, the percentage of absorbance at 470 nm was determined as compared to control wells where an equivalent amount of DMSO had been added. Data are presented as the mean ± standard deviation of experiments performed in triplicate.

To quantify the cellular cytotoxicity of FK778 in growing monolayers of HEL cells, subconfluent cultures were prepared in 24-well plates. Seventy-two hours post incubation

with selected concentrations of FK778, the percent confluence of wells were evaluated by inverted light microscopy and compared to control wells where an equivalent amount of DMSO had been added. Data are represented as the mean ± standard deviation of experiments performed in triplicate.

2.5. Western blot analyses

HEL fibroblasts were grown to confluence, serum-starved for 48 h, and infected with HCMV (Towne strain) at a multiplicity of infection (MOI) of 5 pfu/cell. Cells were either treated or untreated with 32 μM FK778 1 h prior to infection, and drug remained in the media until cells were harvested at the indicated times by scraping monolayers in 2× Laemmli sodium dodecyl sulfate (SDS) sample buffer. In some experiments, various concentrations of uridine were also added to drug-treated and control cultures. Samples were boiled, loaded onto 8% SDS-polyacrylamide gels, and proteins were separated by polyacrylamide gel electrophoresis (PAGE) then transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). Blots were blocked for 1 h in 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS-T). Blots were probed with primary antibodies (1:1000 to 1:30,000 dilutions) for 14 h at 4 °C. Blots were then washed with PBS-T (4 × 50 mL × 10 min) at room temperature then probed with secondary horse radish peroxidase-conjugated antibodies, washed and developed by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Amersham Biosciences Corp., Piscataway, NJ).

2.6. Cell nuclear extract isolations

Extracts were prepared as described previously (Yurochko et al., 1995, 1997a,b). Briefly, mock-infected and infected HEL fibroblasts (with and without 10 μg/mL FK778) were collected by scraping cell monolayers in ice-cold PBS and centrifuging. Cells were resuspended and incubated for 10 min on ice in 50 μL of a cytoplasmic isolation buffer, consisting of 10 mM HEPES (pH 7.6), 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), and aliquots of protease inhibitor cocktail (Sigma). Samples were centrifuged and the cytoplasmic supernatants were removed. The nuclear pellets were washed with cytoplasmic extraction buffer (minus NP-40) and then incubated for 10 min on ice in 25 μL of a nuclear isolation buffer, consisting of 20 mM Tris (pH 8.0), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% v/v glycerol and aliquots of protease inhibitor cocktail (Sigma). Samples were centrifuged and the desired supernatant extracts were stored at −70 °C.

2.7. Electrophoretic mobility shift assays (EMSA)

EMSA were performed as described previously (Yurochko et al., 1997a,b; Yurochko and Huang, 1999). Briefly, equal amounts of nuclear extracts (as determined by colorimetric protein content assays, Bio-Rad; Hercules, CA) were incubated for 10 min at room temperature (RT) in binding buffer (10 mM Tris–HCl, 50 mM NaCl, 7.5 mM $MgCl_2$, 0.5 mM EDTA, 10% v/v glycerol, 1 mM DTT) with 2 μg poly(dI-dC). ^{32}P-labeled double-stranded deoxy oligonucleotide probes were added, and incubated for 10 min at RT. Samples were then electrophoresed on a 5% polyacrylamide gel, dried, and analyzed by autoradiography. Probes were labeled by annealing complimentary deoxy oligonucleotides and filling in overhanging ends with [α-^{32}P]dATP. A wild-type GC box (5′-CCTTTTTAAGGGGCGGGGCTT-3′) probe was used for the experiments examining Sp1 activity, and a wild-type major histocompatibility complex NF-κB binding site (5′-CCTTTTTTTTTGGGGATTCCCCA-3′) probe was used for experiments examining NF-κB activity.

2.8. EGF receptor and PI3-K immunoprecipitation

Ten centimeter dishes of HEL cells, treated and untreated with 10 μg/mL FK778 were harvested at various times post infection as described above. Cytoplasmic extracts were prepared from ice-cold PBS scraped cell monolayers by a 10 min incubation at 0 °C in 0.5 mL of immunoprecipitation (i.p.) buffer containing 240 mM NaCl, 100 mM NaF, 200 mM Na-orthovanadate, 0.5% NP-40, 50 mM Tris, pH 8.0 with protease inhibitor cocktail (Sigma) in Eppendorf tubes. Equivalent input protein content of samples was determined by colorimetric assays (Bio-Rad). Samples were incubated with 12 μL mouse mAb to the EGF receptor (or 5 μL rabbit pAb to PI3-K p85α) with rocking at 4 °C for 3 h, then a 20 μL of a suspension of protein G-sepharose beads (Amersham) was added, and samples were incubated for an additional 8 h at 4 °C with gentle agitation. Samples were centrifuged, the supernatant was removed, and the pellets were washed four times with 4 °C PBS. Beads were boiled in 2× Laemmli buffer and the supernatant was loaded onto polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. The same membrane was analyzed first with an antibody specific for phosphotyrosine then with an antibody specific for total EGF receptor (or PI3-K p85α) by Western blot, as described above.

2.9. HCMV DNA synthesis quantification (dot blot)

Dot blots for the quantification of HCMV DNA synthesis were performed similarly to that described previously (Johnson et al., 1999). HEL cells were grown to confluence in 24-well plates, serum-starved for 48 h, then infected with HCMV Towne at 2–5 pfu/cell in the presence or absence of 32 μM FK778, 100 μM leflunomide, and 50 μM GCV. At 72 h post infection, plates were harvested by removing the cell culture medium and replacing it with 0.2 mL PBS per well, then freezing at −70 °C. Plates were thawed and 0.4 mL per well denaturation buffer consisting of 1.5 M NaCl

and 1 M NaOH was added. After 10 min, 0.4 mL neutralization buffer consisting of 1 M NaCl and 1 M Tris–HCl pH 7.0 was added to wells. After 5 min samples were transferred to an Immobilion-NC membrane (Millipore) with a minifold apparatus (Schleicher & Schuell; Keene, NH) and immobilized under vacuum by baking at 80 °C for 2 h. Non-specific binding was blocked by incubation in 5× Denhardt's 5× SSC, 50% formamide, 1% SDS, with 100 μg/mL salmon sperm DNA at 42 °C for 3 h. The membrane was probed in blocking buffer for 14 h at 42 °C with denatured Towne genomic DNA, that had been labeled with [α-^{32}P]dATP (NEN; Boston, MA) by nick translation according to kit instructions (Invitrogen; Carlsbad, CA). Following washes (twice with 2× SSC + 1% SDS; twice with 0.5× SSC + 1% SDS; once with 0.1× SSC + 1% SDS; rinsed with 6× SSC), the membrane was developed by autoradiography.

3. Results

3.1. Antiviral activity against HCMV and cellular cytotoxicity of FK778

Initial evaluation of FK778 as a potential antiviral revealed that this compound inhibited HCMV replication in plaque reduction assays in a dose-dependent manner (Fig. 2). We determined an antiviral IC_{50} of 1.97 μM for FK778 against the Towne strain (Fig. 2), nearly as potent as was positive control compound GCV. Significantly higher concentrations of FK778 were necessary to produce cellular cytotoxicity in both static and growing HEL cells (Fig. 2). Since there are differences between clinical isolates and some laboratory/vaccine strains of HCMV (Prichard et al., 2001), we measured the ability of FK778 to inhibit clinical isolates of HCMV. The data presented in Table 1 indicate that FK778 inhibited the replication of two clinical isolates at least as potently as it inhibited the Towne strain.

Table 1
Antiviral activity[a] of FK778 against laboratory (Towne) and clinical (Major and TW-087) HCMV isolates

HCMV isolate	IC_{50}[b] (μM)
Towne	1.35–1.97[c]
Major	0.83–1.09 [c]
TW-087	1.04–1.15 [c]

[a] The antiviral activity of FK778 was determined by plaque reduction assay as described in Section 2.

[b] IC_{50}; drug concentration producing 50% inhibition of HCMV plaque formation. Results are reported as the range of at least two independent experiments, each performed in quadruplicate.

[c] In these experiments, GCV was employed as a positive control and reproducibly resulted in approximately 90% inhibition at a fixed concentration of 10 μM.

FK778 potently inhibited HCMV plaque formation with an in vitro selectivity index (cytotoxic IC_{50} divided by antiviral IC_{50}) of at least 100 (Fig. 2). In order to determine the antiviral mode of action for FK778, we chose a drug concentration that potently inhibited viral replication but did not appear to display gross cytotoxicity in cell

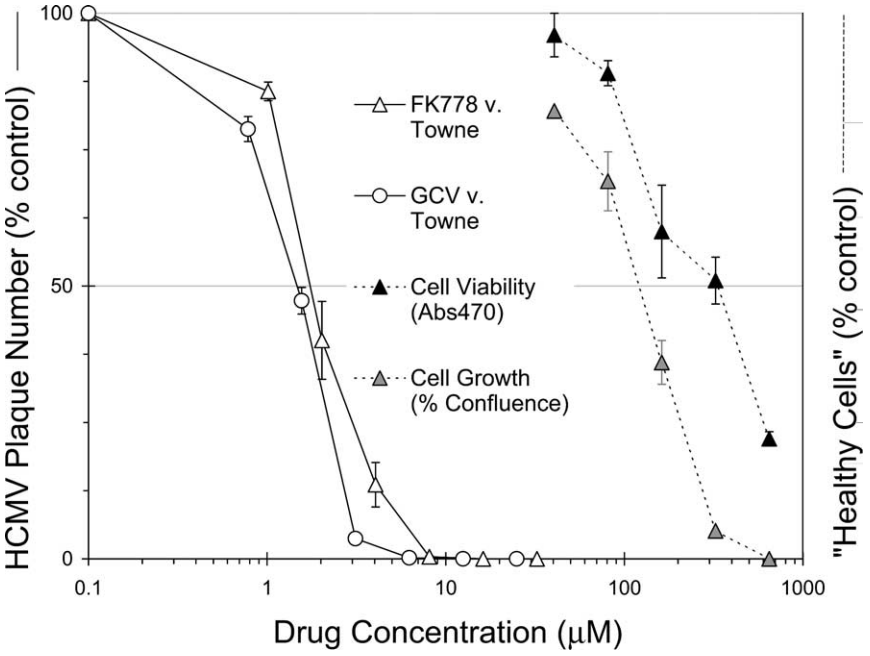

Fig. 2. Antiviral activity (plaque reduction assay) and cytotoxicity of FK778. Plaque reduction assays and cytotoxicity measurements were determined as described in Section 2. Triangles represent FK778, while circles represent GCV (positive control). Solid lines indicate antiviral activity, while dashed lines indicate cytotoxicity measurements. Data are represented as the mean ± standard deviation of experiments performed in duplicate (GCV) or quadruplicate (FK778).

culture. All subsequent experiments were performed at 32 μM (10 μg/mL) FK778.

3.2. Effects of FK778 on HCMV-mediated signal transduction

We next examined the ability of FK778 to block HCMV-induced tyrosine phosphorylation of the EGF receptor. Immunoprecipitation followed by Western blotting demonstrated similar levels of total EGF receptor protein levels in mock-infected cells as compared to virus-infected cells (Fig. 3a). Consistent with previously reported results (Wang et al., 2003), HCMV-induced EGF receptor tyrosine autophosphorylation was detected from 10 to 60 min post infection. Hyperphosphorylated species of EGFR were reproducibly absent upon treatment with FK778 at ten minutes post infection, but inhibition decreased over time, until no inhibition was detected from 30 min to 1 hpi.

To determine the effect(s) of FK778 upon HCMV activation of PI3-K, we compared the phosphorylated (activated) forms of PI3-K and downstream kinases Akt and p70S6K, to total protein levels of each kinase, in the presence and absence of drug, at various times post infection. Similar to results reported previously (Johnson et al., 2001), infection with HCMV had minimal effect on the total protein levels of PI3-K, Akt, and p70S6K but increased in their phosphorylation. FK778 partially inhibited the first phase of HCMV-mediated PI3-K induction (Fig. 3b). Inhibition of this phase of PI3-K signaling was not transient and continued to a greater or lesser extent through 80 min, the latest time point examined. The second phase (12–48 hpi) of HCMV-mediated PI3-K activation was more sensitive to FK778 treatment, as reflected by the hypophosphorylated forms of Akt, p70S6K, and p85 (Fig. 3b).

NF-κB and Sp1 activate the major IE gene promoter (MIEP) of HCMV and are probably necessary for continuation of the viral gene expression cascade (Cherrington and Mocarski, 1989; Sambucetti et al., 1989). Consistent with HCMV-mediated PI3-K signaling, analysis of the effects of FK778 upon HCMV-induced signal transduction pathways by EMSA revealed that this compound partially inhibited the initial phase of NF-κB and Sp1 activation (Fig. 4). From the IE phase of HCMV infection, the activities of NF-κB and Sp1 were slightly inhibited by this compound. However, from 12 to 48 hpi the HCMV-mediated activities of NF-κB and Sp1 were reduced to background upon treatment with

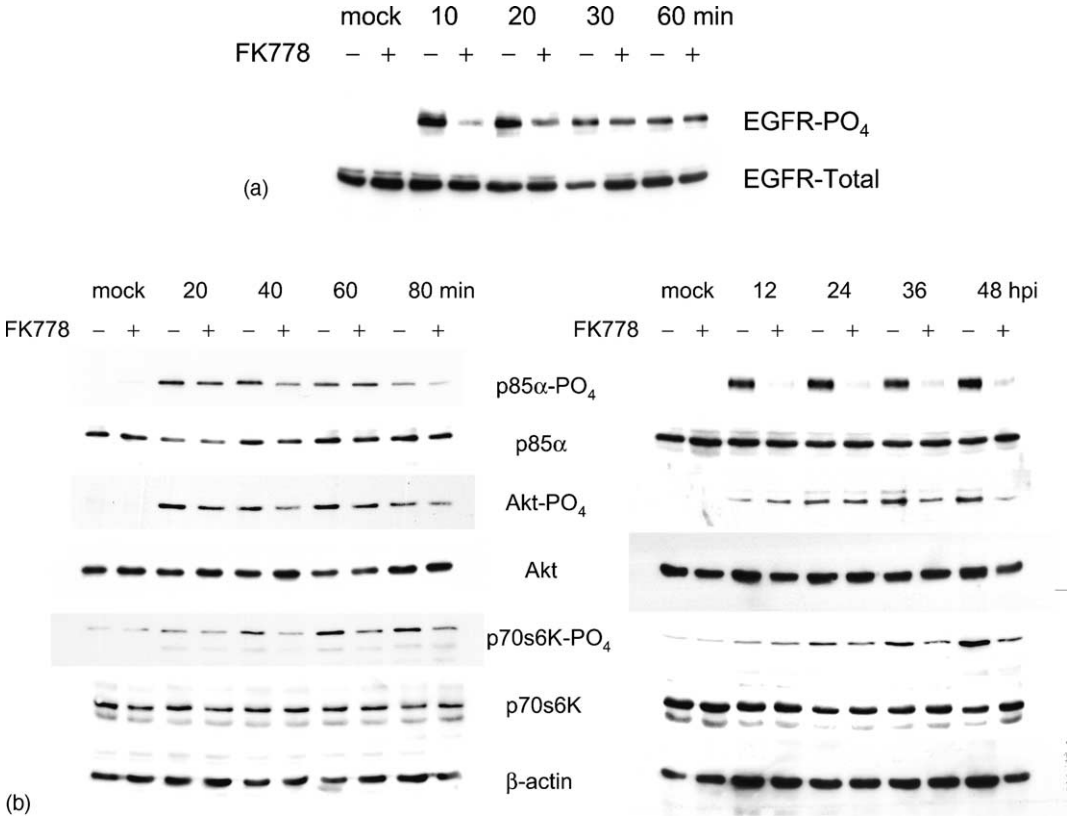

Fig. 3. HCMV-specific EGF receptor phosphorylation and PI3-K activation in the presence and absence of 32 μM FK778. (a) Immunoprecipitation of the EGF receptor was followed by Western blots for phosphotyrosine and total EGF receptor. (b) The first and second phases of PI3-K activation by HCMV were determined by Western blots of whole cell lysates for total and phosphorylated Akt (Ser 473) and p70S6K (Thr 389), total and phosphorylated PI3-K p85 were determined by immunoprecipitation of p85, followed by Western blotting for phosphotyrosine and the p85 subunit of PI3-K. Control β-actin levels indicated that similar levels of protein were loaded in each lane.

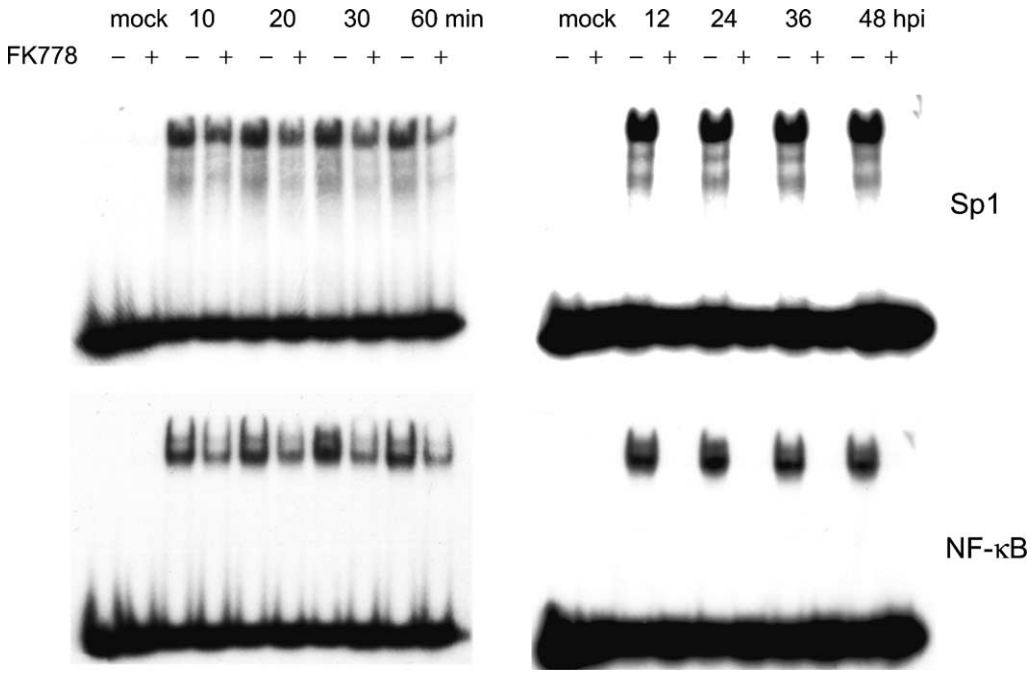

Fig. 4. Effects of FK778 upon both phases of HCMV-specific NF-κB and Sp1 induction as measured by EMSA: (a) NF-κB binding to an MHC class I probe; (b) Sp1 binding to a GC box.

FK778 (Fig. 4). Thus, FK778 inhibited the first and second tiers of HCMV-specific NF-κB and Sp1 induction in a manner similar to its inhibition of the phosphorylation of PI3-K p85, Akt, and p70S6K.

3.3. Effects of FK778 upon HCMV protein levels

In order to identify the stage(s) of HCMV replication that were specifically inhibited by FK778, we measured its effects upon viral proteins at various times post infection by Western blot analyses. FK778 demonstrated limited inhibition of IE1-72 levels (Fig. 5a). In contrast, the levels of the second HCMV major immediate-early protein were dramatically altered by FK778. Treatment of infected cells with FK778 resulted in no inhibition of IE2-86 at 12 hpi. Levels of IE2-86 in the presence of FK778 were decreased at 24 hpi and reached undetectable levels at 48 and 72 hpi (Fig. 5a). We reproduced the different effects of FK778 upon IE1-72 and IE2-86 with control compound GCV (Fig. 5b). The levels of early late (UL84) (He et al., 1992) and true late (UL94) (Wing et al., 1996) gene products were similarly inhibited by treatment with FK778 (Fig. 5a), reduced to undetectable levels in the presence of drug at 48 and 72 hpi. Noting that IE1-72 and gB were the only viral protein levels measured to determine that leflunomide did not inhibit HCMV protein synthesis (Waldman et al., 1999a), we also measured the levels of gB, and performed a Western blot using human hyperimmune globulin (Cytogam) to HCMV as a primary antibody. Although there are numerous undefined HCMV-specific antigenic determinants in Cytogam (Snydman, 2001), this experiment provided some measure of confidence that the effects observed were representative of HCMV protein expression in general. In contrast to the results reported for the parent compound leflunomide, we determined that not only are levels of gB reduced to background in the presence of FK778, but also that the levels of all virus-specific antigenic proteins measurable with Cytogam were potently inhibited at 48 and 72 hpi, but not at 12 hpi. Thus treatment with FK778 resulted in reduced levels of HCMV early and late proteins.

3.4. Effects of FK778 upon HCMV DNA and comparison to leflunomide

While it was reported that leflunomide did not inhibit HCMV protein synthesis or DNA replication (Waldman et al., 1999a; Knight et al., 2001), the above results indicated that FK778 probably caused a general block to the synthesis of early and late viral proteins. By definition, late genes in the lytic replication cycle of HCMV are those that can only be expressed after the replication of viral DNA (Huang, 1975a). This motivated us to examine the effects of FK778 and leflunomide upon HCMV DNA replication. Fig. 6 shows that HCMV-specific DNA was reduced in a dose-dependent manner in infected cells treated with FK778. Leflunomide reproducibly reduced HCMV DNA levels but was not as potent an inhibitor as was FK778 or positive control compound GCV.

Plates identical to those prepared for dot blots were examined at 96 h post infection for HCMV titer content. We determined that leflunomide had an IC_{50} of 11 ± 2 μM and inhibited HCMV replication by at least 75% at concentrations

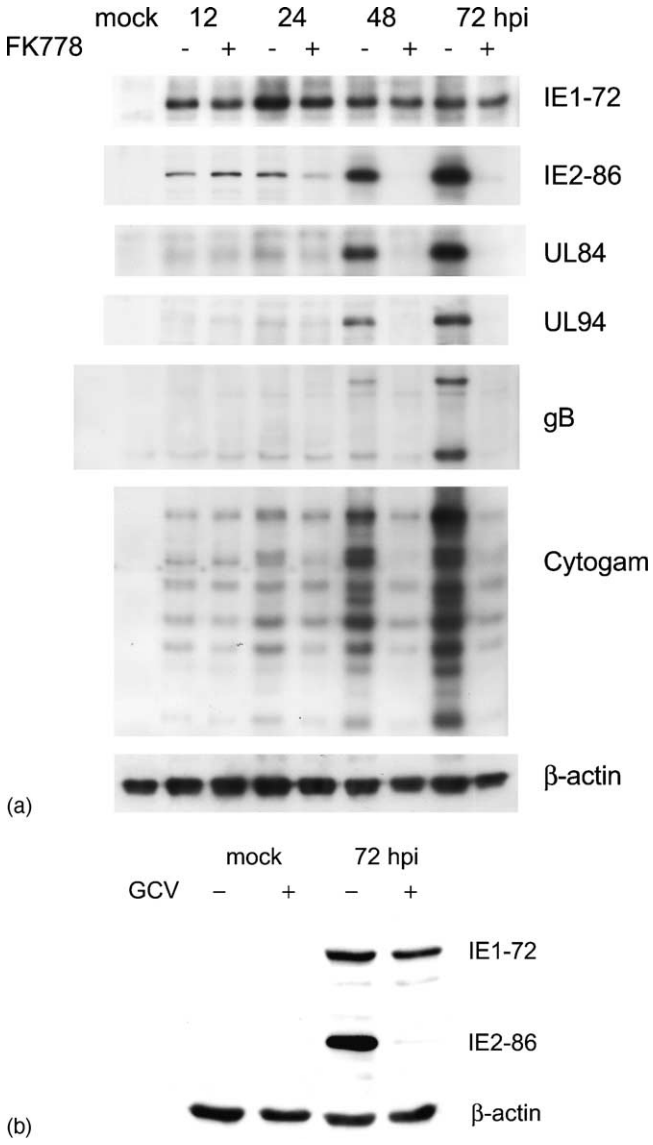

Fig. 5. The effects of FK778 on HCMV proteins. (a) HCMV protein levels at various time points in the presence and absence of 32 μM FK778 were determined by Western blots. Control β-actin levels indicated that similar levels of protein were loaded in each lane. (b) At 72 h post infection, 70 μM GCV, a control HCMV DNA polymerase inhibitor (Mar et al., 1983, 1985), displayed the same pattern of inhibition of IE1-72 and IE2-86 as did FK778.

≥25 μM (data not shown). Also, 200 μM leflunomide displayed visible cytotoxicity against static HEL cells, while concentrations less than or equal to 100 μM did not (data not shown).

3.5. Antiviral activity of FK778 was reversed by treatment with uridine

Since HCMV DNA replication was blocked by FK778, it seemed possible that its antiviral mechanism(s) might be consistent with the previously described mode of immunosuppression described for leflunomide: deprivation of de

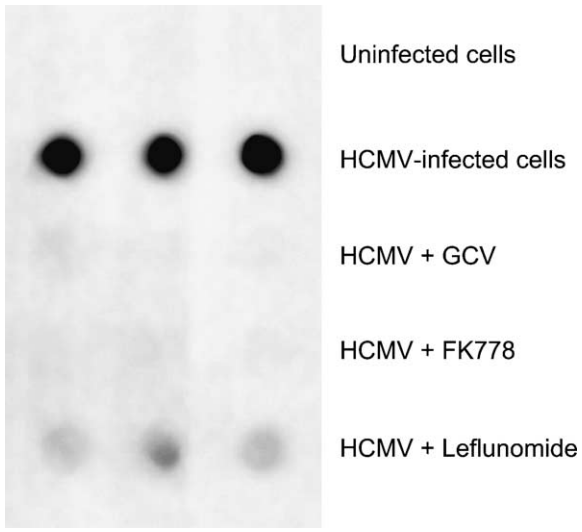

Fig. 6. Effects of FK778 and leflunomide upon HCMV DNA levels. Bottom two rows: HCMV DNA levels in infected cells treated with FK778 (32 μM) or leflunomide (100 μM) were determined by dot (Southern) blotting. Top three rows: controls are shown for uninfected cells, HCMV-infected cells, and HCMV-infected cells treated with 50 μM GCV. Data are shown in triplicate.

novo biosynthesis of pyrimidines via inhibition of DHODH. To test this hypothesis, we examined the ability of uridine to reverse the antiviral activity of FK778 in a titer reduction assay. Here we employed high MOI conditions (2–5 PFU/cell) with a fixed concentration of drug (no drug control, 32 μM FK778, 70 μM GCV positive control) while varying concentrations of exogenous uridine (Fig. 7). The antiviral activity of FK778 was reversed by uridine in a dose-dependent manner, reproducibly decreasing the ability of this compound to reduce HCMV titers by approximately 3–4 orders of magnitude. A 100 μM uridine treatment fully reversed the antiviral activity of FK778. In contrast, GCV displayed similar inhibition of HCMV at all concentrations of uridine tested.

4. Discussion

We have examined the possibility that FK778 might possess antiviral activity against HCMV, and found that FK778 possessed potent and selective activity against HCMV. It was determined elsewhere that FK778 inhibited mitogen-activated T cell proliferation in cell culture with an IC_{50} of 102 mM (32 μg/mL) in human whole blood samples (Gregory et al., 1998). Safe and tolerated in vivo therapeutic serum trough levels for FK778 in canines were reported to range from 40 to 100 μg/mL (Jin et al., 2002), some 30–100 times the in vitro IC_{50}s we determined against HCMV. Although our work in conjunction with these studies does not present a direct comparison between immunosuppression and antiviral activity under identical conditions, it seems reasonable to expect that patients treated with FK778 for immunosuppression may benefit from concomitant

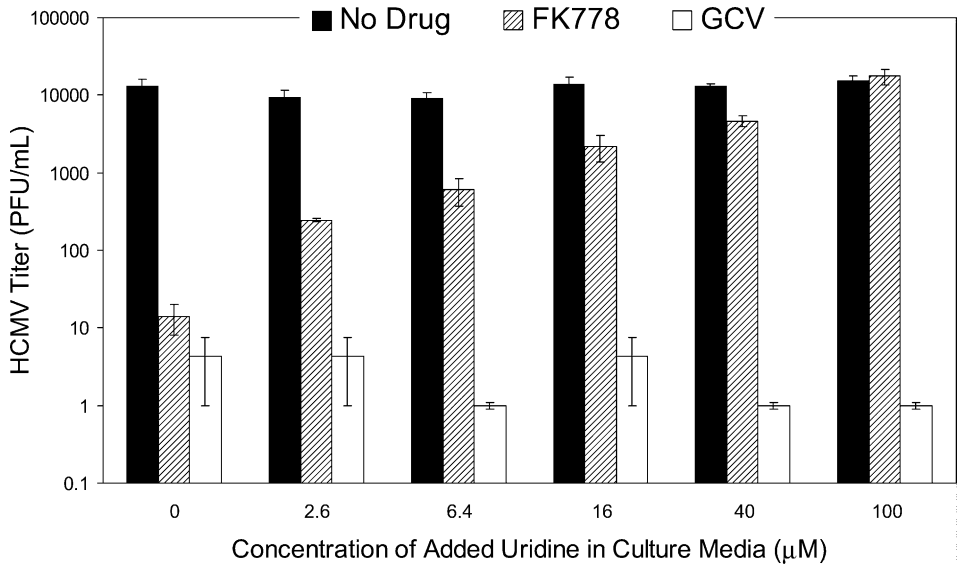

Fig. 7. Uridine reverses the antiviral activity of FK778. For each amount of added uridine, HCMV (Towne strain) titer production was determined in the absence of drug (virus/negative control; filled bars), with 32 μM FK778 (lined bars), and with 70 μM GCV (positive control; open bars). Data are presented as the mean ± standard deviation of experiments performed in quadruplicate.

inhibition of HCMV replication. Similarly, FK778 was recently shown to protect blood vessels in an animal model of endothelial injury, suggesting additional promise for this drug in the treatment of restenosis and chronic allograft rejection (Savikko et al., 2003).

The second aim of this study was to determine how FK778 inhibited HCMV replication. FK778 exhibited transient inhibition of HCMV-induced phosphorylation of the EGF receptor, as well as inhibition of both phases of HCMV-mediated PI3-K signaling and Sp1 and NF-κB activities. However, we have not ruled out the induction of phosphatase(s) or other less direct mechanisms for these results. It is possible that a minor reduction in the first phase of HCMV-mediated signal transduction (Fig. 3a and b) could produce a large effect upon subsequent HCMV replication. However, we suspect that this is not the primary mode of action for FK778 against HCMV for two reasons. First, our results (Figs. 6 and 7) indicated dose-dependent and complete reversal of FK778's antiviral effects by treatment with uridine. We are unaware of any reports describing uridine as a modulator of EGF receptor phosphorylation, PI3-K signaling, or Sp1 and NF-κB expression or DNA binding. Second, Western blot analyses indicated the expression of viral immediate-early proteins in the presence of FK778, indicating that this compound most likely did not prevent HCMV from entering cells.

Early in vitro studies on the biochemical basis for the immunosuppressive properties of leflunomide indicated that it inhibited protein tyrosine kinases. However, such inhibition required concentrations of 30–100 μM A771726 (Mattar et al., 1993; Xu et al., 1995). In contrast, IC_{50} values determined for the inhibition of purified DHODH by A771726 ranged from 10 to 30 nM (Williamson et al., 1995; Davis et al., 1996). The differences in potency against the two targets caused some to conclude that the primary target was DHODH. It is possible that using a fixed concentration of FK778 greater than its antiviral IC_{50} in order to inhibit viral replication by nearly 100%, produced inhibition of early HCMV-mediated signal transduction as a secondary artifact. However, at the plasma concentrations of FK778 required for immunosuppression, it is likely that the contribution(s) of HCMV-mediated signal transduction to the pathogenicity of HCMV infection will be at least attenuated.

Western blot analyses indicated a general block by FK778 to the synthesis of early and late viral proteins. The differences in inhibition of the major immediate early viral proteins IE1-72 and IE2-86 might appear to preclude a similar simplification regarding the general effect(s) of FK778 on the synthesis of immediate early viral proteins. Although IE1-72 and IE2-86 share the same promoter (reviewed in Castillo and Kowalik, 2002), there are numerous differences between these two proteins. For example, IE1-72 is an immediate early gene, while IE2-86 is both an immediate early and apparently also a true late gene (sensitive to GCV) (Chambers et al., 1999). We also note that FK778's alteration of HCMV signal transduction may contribute to these effects. An apparent requirement for NF-κB in the transactivation of the major immediate-early promoter has been described (Cherrington and Mocarski, 1989; Sambucetti et al., 1989). IE1-72 is dispensable for HCMV replication under high MOI conditions (Greaves and Mocarski, 1998) such as those employed for the Western blots in this report. In contrast, IE2-86 is apparently required for productive viral infection (Marchini et al., 2001). While IE1-72 and IE2-86 are generally regarded as the canonical immediate early HCMV proteins, their expression continues through the entire viral replication cycle and the expression of IE2-86, but

not IE1-72, increases as viral replication proceeds (Speir et al., 1994). Considering that we determined a similar inhibitory pattern for IE1-72 and IE2-86 with GCV and FK778 (Fig. 5b), it is possible that poor inhibition of IE1-72 concurrent with strong inhibition of IE2-86 at 48–72 h post infection resulted from the aftermath of a general block to the synthesis of HCMV early/late proteins. Western blots measure protein amounts, but do not indicate whether changes in amounts are due to altered protein synthesis or altered stability/degradation. It is thus also possible that an extended intracellular half-life for IE1-72 is responsible for its apparent lack of inhibition by FK778 and GCV.

The simplest explanation for the uridine reversal of inhibition of HCMV by FK778 is that uridine shifts the supply of pyrimidine building blocks for cellular and viral DNA and RNA from the de novo to the salvage pathways. If this is the case, then it follows that the replication of HCMV is more sensitive (approximately 100-fold) to de novo pyrimidine biosynthesis deprivation than are its presumably quiescent host cells. Qualitative support for this hypothesis is provided by a report that HCMV replication proceeds with an approximately 15–30-fold increase in cellular nucleotide pools as compared to uninfected cells (Biron et al., 1986). This evidence leads us to hypothesize that the primary molecular target for FK778 may be DHODH and that the major antiviral effects of FK778 may be mediated through depriving cells of pyrimidines. Further studies will be required to validate or invalidate this hypothesis. The same evidence (uridine reversal) does not detract from the alternative hypothesis that FK778 might directly inhibit the salvage pathway of pyrimidine biosynthesis. It is also possible that employment of alternate conditions could shift the inhibition of HCMV-induced signal transduction to a different extent (higher level). We did not attempt to compete out FK778 with the addition of excess substrate for DHODH because related malononitriloamide inhibitors did not compete with substrate for catalysis (Williamson et al., 1995). It might be useful to determine whether or not a panel of genuinely specific structurally distinct small molecule inhibitors of DHODH possesses similar selective antiviral activity against HCMV and other herpesviruses.

This is not the first report where the basis of a compound's antiviral activity has been traced to the apparent inhibition of a cellular enzyme or a cellular pathway. Inhibitors of cellular cyclin-dependent kinases were active against HSV-1 (Schang et al., 1998) and HCMV (Bresnahan et al., 1997). Therapeutic compounds active against cellular cyclooxygenase 2 inhibited the replication of HCMV (Speir et al., 1998). Inhibition of cellular p38 mitogen-activated protein kinase demonstrated selective inhibition of HCMV (Johnson et al., 1999). Casein kinase II has been proposed to mediate the antiviral affects of certain inhibitors of HIV-1 replication (Critchfield et al., 1997). However, pivotal proof of a genuinely selective cellular antiviral target has yet to be demonstrated.

Given their structural similarities, it was surprising that our determination of the mode of antiviral action of FK778 was different than that determined by others (Waldman et al., 1999a,b; Knight et al., 2001) for the parent compound leflunomide/A771726. Structural similarity is no guarantee of identical biochemical mechanism of action. For example, radically different modes of antiviral action were shown for two structurally similar aryl thiourea compounds. WAY-150138 inhibited HSV DNA cleavage and maturation (van Zeijl et al., 2000), while CF102 inhibited HCMV fusion/entry (Jones et al., 2004). Regardless, we propose three possible explanations for the differences between the data presented in this study and the antiviral mechanism described for A771726 against HCMV and HSV (Waldman et al., 1999a,b; Knight et al., 2001). First, our examination of FK778 was against the Towne strain of HCMV in HEL cells. In contrast, they studied A771726 against the P8 and VHL/E strains of HCMV in human foreskin fibroblasts and in human umbilical vein endothelial cells, respectively (Waldman et al., 1999a). Second, FK778 (IC_{50} 1–2 μM) and leflunomide (IC_{50} 11 μM) were more potent inhibitors in our assays than was described for A771726 (IC_{50} 40–60 μM) (Waldman et al., 1999a). They studied the effects of a drug concentration that was ∼2 times the IC_{50} of A771726, while we studied the effects of a drug concentration that was ∼20 times the IC_{50} of FK778 and ∼9 times the IC_{50} of leflunomide, upon HCMV replication. Some antiviral effects may not have been apparent when evaluated against the weaker drug pressure they employed. In support of this explanation, one study reported two antiviral mechanisms of action for leflunomide against HIV. Drug concentration alone determined whether or not the effects of leflunomide against HIV were reversible by uridine (Schlapfer et al., 2003). Third, though less likely, it remains possible that our cursory evaluation of leflunomide failed to reflect the antiviral biology of its metabolite, A771726. We did not assume or attempt to measure rapid nonenzymatic conversion of leflunomide to A771726 in cell culture media at 37 °C. We conclude that there exists some uncertainty as to the "true" antiviral mode of action against HCMV for FK778 and leflunomide/A771726. However, it is certain that this study agrees with the potential positive impact of this class of immunosuppressives upon what might otherwise have been an invitation for opportunistic infections (Waldman et al., 1999b).

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References

Andreoni, K.A., Wang, X., Huong, S.M., Huang, E.-S., 2002. Human cytomegalovirus hyperimmune globulin not only neutralizes HCMV infectivity, but also inhibits HCMV-induced intracellular NF-κB, Sp1, and PI3-K signaling pathways. J. Med. Virol. 67, 33–40.

Biron, K.K., Stanat, S.C., Sorrell, J.B., Fyfe, J.A., Keller, P.M., Lambe, C.U., Nelson, D.J., 1986. Metabolic activation of the nucleoside analog 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc. Natl. Acad. Sci. U.S.A. 82, 2473–2477.

Bresnahan, W.A., Boldogh, I., Chi, P., Thompson, E.A., Albrecht, T., 1997. Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. Virology 231, 239–247.

Castillo, J.P., Kowalik, T.F., 2002. Human cytomegalovirus immediate-early proteins and cell growth control. Gene 290, 19–34.

Chambers, J., Angulo, A., Amaratunga, D., Guo, H., Jiang, Y., Wan, J.S., Bittner, A., Frueh, K., Jackson, M.R., Peterson, P.A., Erlander, M.G., Ghazal, P., 1999. DNA microarrays of the complex human cytomegalovirus genome: profiling kinetic class with drug sensitivity of viral gene expression. J. Virol. 73, 5757–5766.

Cherrington, J.M., Mocarski, E.S., 1989. Human cytomegalovirus IE1 transactivates the a promoter-enhancer via an 18-base-pair repeat element. J. Virol. 63, 1435–1440.

Chong, A.S.F., Huang, W., Liu, W., Luo, J., Shen, J., Xu, W., Ma, L., Blinder, L., Xiao, F., Xu, X., Clardy, C., Preston Foster, P., Williams, J.A., 1999. In vivo activity of leflunomide: pharmacokinetic analyses and mechanism of immunosuppression. Transplantation 68, 100–109.

Critchfield, J.W., Coligan, J.E., Folks, T.M., Butera, S.T., 1997. Casein kinase II is a selective target of HIV-1 transcriptional inhibitors. Proc. Natl. Acad. Sci. U.S.A. 94, 6110–6115.

Davis, J.P., Cain, G.A., Pitts, W.J., Magolda, R.L., Copeland, R.A., 1996. The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. Biochemistry 35, 1270–1273.

Erice, A., 1999. Resistance of human cytomegalovirus to antiviral drugs. Clin. Microbiol. Rev. 12, 286–297.

Greaves, R.F., Mocarski, E.S., 1998. Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus *ie1* mutant. J. Virol. 72, 366–379.

Griffiths, P.D., 2002. The treatment of cytomegalovirus infection. J. Antimicrob. Chemother. 49, 243–253.

Gregory, C.R., Silva, H.T., Patz, J.D., Morris, R.E., 1998. Comparative effects of malononitriloamide analogs of leflunomide on whole blood lymphocyte stimulation in humans, rhesus macaques, cats, dogs, and rats. Transpl. Proc. 30, 1047–1048.

He, Y.S., Xu, L., Huang, E.-S., 1992. Characterization of human cytomegalovirus UL84 early gene and identification of its putative protein product. J. Virol. 66, 1098–1108.

Huang, E.-S., 1975a. Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. 16, 298–310.

Huang, E.-S., 1975b. Human cytomegalovirus IV. Specific inhibition of virus induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. 16, 1560–1565.

Huang, E.-S., Kilpatrick, B.A., Huang, Y.T., Pagano, J.S., 1976. Detection of human cytomegalovirus and analysis of strain variation. Yale J. Biol. Med. 49, 29–43.

Huang, E.-S., Chen, S.T., Pagano, J.S., 1973. Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. 12, 1473–1481.

Jin, M.B., Nakayama, M., Ogata, T., Fujita, M., Mino, K., Taniguchi, M., Suzuki, T., Shimamura, T., Furukawa, H., Todo, S., 2002. A novel leflunomide derivative, FK778, for immunosuppression after kidney transplantation in dogs. Surgery 132, 72–79.

Johnson, R.A., Huong, S.-M., Huang, E.-S., 1999. Inhibitory effect of 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole on HCMV DNA replication and permissive infection. Antiviral Res. 41, 101–111.

Johnson, R.A., Wang, X., Ma, X.L., Huong, S.-M., Huang, E.-S., 2001. Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. J. Virol. 75, 6022–6032.

Jones, T.R., Lee, S.W., Johann, S.V., Razinkov, V., Visalli, R.J., Feld, B., Bloom, J.D., O'Connell, J., 2004. Specific inhibition of human cytomegalovirus glycoprotein B-mediated fusion by a novel thiourea small molecule. J. Virol. 78, 1289–1300.

Knight, D.A., Hejmanoswki, A.Q., Dierksheide, J.E., Williams, J.W., Chong, A.S.F., Waldman, W.J., 2001. Inhibition of herpes simplex virus type 1 by the experimental immunosuppressive agent leflunomide. Transplantation 71, 170–174.

Kuo, E.A., Hambleton, P.T., Kay, D.P., Evans, P.L., Matharu, S.S., Little, E., McDowall, N., Jones, C.B., Hedgecock, C.J.R., Yea, C.M., Chan, A.W.E., Hairsine, P.W., Ager, I.R., Tully, W.R., Williamson, R.A., Westwood, R., 1996. Synthesis, structure-activity relationships and pharmacokinetic properties of dihydroorotate dehydrogenase inhibitors: 2-cyano-3-cyclopropyl-3-hydroxy-*N*-[3′-methyl-4′-(trifluoromethyl)phenyl]propenamide and related compounds. J. Med. Chem. 39, 4608–4621.

Landolfo, S., Gariglio, M., Gribaudo, G., Lembo, D., 2003. The human cytomegalovirus. Pharmacol. Ther. 98, 269–297.

Manna, S.K., Mukhopadhyay, A., Aggarwal, B.B., 2000. Leflunomide suppresses TNF-induced cellular responses: effects of NF-κB, activator protein-1, c-Jun N-terminal protein kinase, and apoptosis. J. Immunol. 165, 5962–5969.

Mar, E.C., Cheng, Y.C., Huang, E.-S., 1983. Effect of 9-(13-dihydroxy-2-propoxymethyl)guanine on human cytomegalovirus replication in vitro. Antimicrob. Agents Chemother. 24, 518–521.

Mar, E.C., Chiou, J.F., Cheng, Y.C., Huang, E.-S., 1985. Inhibition of cellular DNA polymerase alpha and human cytomegalovirus-induced DNA polymerase by the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 9-(13-dihydroxy-2-propoxymethyl)guanine. J. Virol. 53, 776–780.

Marchini, A., Liu, H., Zhu, H., 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J. Virol. 75, 1870–1878.

Mattar, T., Kochhar, K., Bartlett, R., Bremer, E.G., Finnegan, A., 1993. Inhibition of the epidermal growth factor receptor tyrosine kinase activity by leflunomide. FEBS Lett. 334, 161–164.

Plotkin, S.A., 1999. Vaccination against cytomegalovirus, the changeling demon. Ped. Inf. Dis. J. 18, 313–326.

Perry, C.M., Barman Balfour, J.A., 1999. Fomivirsen. Drugs 57, 375–380.

Prichard, M.N., Turk, S.E., Coleman, L.A., Engelhardt, S.L., Shipman Jr, C.S., Drach, J.C., 1990. A microtiter virus yield reduction assay for the evaluation of antiviral compounds against human cytomegalovirus and herpes simplex virus. J. Virol. Meth. 28, 101–106.

Prichard, M.N., Penfold, M.E.T., Duke, G.M., Spaete, R.R., Kemble, G.W., 2001. A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. Rev. Med. Virol. 11, 191–200.

Rawlinson, W., Scott, G., 2003. Cytomegalovirus. A common virus causing serious disease. Aust. Fam. Physician 32, 789–793.

Sambucetti, L.C., Cherrington, J.M., Wilkinson, G.W., Mocarski, E.S., 1989. NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. 8, 4251–4258.

Sanders, S., Harisdangkul, V., 2002. Leflunomide for the treatment of rheumatoid arthritis and autoimmunity. Am. J. Med. Sci. 323, 190–193.

Savikko, J., Von Willebrand, E., Hayry, P., 2003. Leflunomide analogue FK778 is vasculoprotective independent of its immunosuppressive ef-

fect: potential applications for restenosis and chronic rejection. Transplantation 76, 455–458.

Schang, L., Phillips, J., Schaffer, P.M., 1998. Requirement for cellular cyclin-dependent kinases in herpes simplex replication and transcription. J. Virol. 72, 5626–5637.

Schlapfer, E., Fischer, M., Ott, P., Speck, R.F., 2003. Anti-HIV-1 activity of leflunomide: a comparison with mycophenolic acid and hydroxyurea. AIDS 17, 1613–1620.

Scholz, M., Doerr, H.W., Cinatl, J., 2003. Human cytomegalovirus retinitis: pathogenecity, immune evasion and persistence. Trends Microbiol. 11, 171–178.

Scott, J.W., DeJoy, S.Q., Jeyaseelan Sr, R., Powell, D.W., Raventos-Suarez, C., O'Hara, B., Wick, M.M., Oronsky, A.L., Kerwar, S.S., 1993. Studies on the effect of CL 306,293, a substituted quinoline carboxylic acid, on the clinical disease induced in mice with LP-BM5 virus. Antiviral Res. 20, 71–81.

Snydman, D.R., 2001. Historical overview of the use of cytomegalovirus hyperimmune globulin in organ transplantation. Transpl. Inf. Dis. 3 (Suppl. 2), 6–13.

Speir, E., Modali, R., Huang, E.-S., Leon, M.B., Shawl, F., Finkel, T., Epstein, S.E., 1994. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 265, 391–394.

Speir, E., Yu, Z.-X., Ferrans, V.J., Huang, E.-S., Epstein, S.E., 1998. Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. Circ. Res. 83, 210–216.

Thomson, T.A., Spinella-Jaegle, S., Francesconi, E., Meakin, C., Millet, S., Flao, K.L., Hidden, H., Ruuth, E., 2002. In vitro and in vivo inhibition of immunoglobulin secretion by the immunosuppressive compound HR325 is reversed by exogenous uridine. Scand. J. Immunol. 56, 35–42.

van Zeijl, M., Fairhurst, J., Jones, T.R., Vernon, S.K., Morin, J., LaRocque, J., Feld, B., O'Hara, B., Bloom, J.D., Johann, S.V., 2000. Novel class of thiourea compounds that inhibit herpes simplex virus type 1 DNA cleavage and encapsidation: resistance maps to the UL6 gene. J. Virol. 74, 9054–9061.

Waldman, W.J., Knight, D.A., Lurain, N.S., Miller, D.M., Sedmak, D.D., Williams, J.W., Chong, A.S.F., 1999a. Novel mechanism of inhibition of cytomegalovirus by the experimental immunosuppressive agent leflunomide. Transplantation 68, 814–825.

Waldman, W.J., Knight, D.A., Blinder, L., Shen, J., Lurain, N.S., Miller, D.M., Sedmak, D.D., Williams, J.W., Chong, A.S.F., 1999b. Inhibition of cytomegalovirus in vitro and in vivo by the experimental immunosuppressive agent leflunomide. Intervirology 42, 412–418.

Wang, X., Huong, S.-M., Chiu, M.L., Raub-Traab, N., Huang, E.-S., 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature 424, 456–461.

Wentworth, B.B., French, L., 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. 135, 253–258.

Williams, J.W., Mital, D., Chong, A., Kottayil, A., Millis, M., Longstreth, J., Huang, W., Brady, L., Jensik, S., 2002. Experiences with leflunomide in solid organ transplantation. Transplantation 73, 358–366.

Williamson, R.A., Yea, C.M., Robson, P.A., Curnock, A.P., Gadher, S., Hambleton, A.B., Woodward, K., Bruneau, J.M., Hambleton, P., Moss, D., Thomson, T.A., Spinella-Jaegle, S., Morand, P., Courtin, O., Sautes, C., Westwood, R., Hercend, T., Kuo, E.A., Ruuth, E., 1995. Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. J. Biol. Chem. 270, 22647–22672.

Wing, B.A., Lee, G.Y.C., Huang, E.-S., 1996. Human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. J. Virol. 70, 3339–3345.

Xu, X., Williams, J., Bremer, E., Finnegan, A., Chong, A., 1995. Inhibition of protein tyrosine phosphorylation in T cells by novel immunosuppressive agent, leflunomide. J. Biol. Chem. 270, 12398–12403.

Yurochko, A.D., Kowalik, T.F., Huong, S.-M., Huang, E.-S., 1995. Human cytomegalovirus upregulates NF-κB activity by transactivating the NF-κB p105/p50 and p65 promoters. J. Virol. 69, 5391–5400.

Yurochko, A.D., Mayo, M.W., Poma, E.E., Baldwin Jr, A.S., Huang, E.-S., 1997a. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-κB promoters. J. Virol. 71, 4638–4648.

Yurochko, A.D., Hwang, E.S., Rasmussen, L., Keay, S., Periera, L., Huang, E.-S., 1997b. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-κB during infection. J. Virol. 71, 5051–5059.

Yurochko, A.D., Huang, E.-S., 1999. Human cytomegalovirus binding to human monocytes induces immunoregulatory gene expression. J. Immunol. 162, 4806–4816.