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The thymidylate synthase inhibitor ZD1694 potently inhibits murine and human cytomegalovirus replication in quiescent fibroblasts

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Abstract

Tomudex (ZD1694) is a quinazoline-based folate analog and a powerful inhibitor of cellular thymidylate synthase and is approved in Europe for use in oncology. Here the first evidence of its activity against murine and human cytomegalovirus (MCMV and HCMV) is reported. ZD1694 irreversibly inhibited the replication and DNA synthesis of both viruses in quiescent fibroblasts. The corresponding 50% effective concentrations were 0.006 and 0.002 μM respectively, whereas the 50% cytotoxic concentration was >10 μM for both murine and human quiescent fibroblasts. A similar antiviral effect was observed against two ganciclovir-resistant HCMV strains isolated from AIDS patients. Taken as a whole these results demonstrate that cellular thymidylate synthase plays an essential role in viral replication and that ZD1694 merits further investigation as anticytomegaloviral agent. © 2000 Published by Elsevier Science B.V.

Keywords: Cytomegalovirus; Thymidylate synthase; Antifolate; ZD1694; Ganciclovir

1. Introduction

Human cytomegalovirus (HCMV) is a common infectious agent of worldwide occurrence and important clinical significance that causes mild or subclinical disease in immunocompetent adults, but can lead to severe morbidity or mortality in neonates or immunocompromised individuals, such as organ transplant recipients and AIDS patients (Ho, 1991). Infections are currently managed by ganciclovir (GCV), foscarnet, or cidofovir treatment (Collaborative DHPG Treatment Study Group, 1986; Jacobson et al., 1991; Studies of Ocular Complications of AIDS Research Group, 1994; Lalezari, 1997). However, drug toxicity and the emergence of resistant virus strains associated with prolonged maintenance therapy have limited their effectiveness (Erice et al., 1989; Drew et al.,

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1991; Knox et al., 1991; Baldanti et al., 1996), and the search for new pharmacological targets for an effective anti-CMV therapy is a major goal.

Unlike others members of the Herpesviridae family, CMV does not encode nucleotide synthesis enzymes such as thymidine kinase (TK), dihydrofolate reductase (DHFR), thymidylate synthase (TS) or an active form of ribonucleotide reductase (RR) (Rawlinson et al., 1996), and thus must mainly rely upon the host cell to supply deoxynucleoside triphosphate (dNTP) and enzyme activities for the synthesis of its DNA. Since folates act as essential cofactors in dNTP de novo synthesis, antifolate-based drugs have been tested for anti-CMV activity. The authors and others have previously observed that methotrexate (MTX), a competitive inhibitor of DHFR, is a potent inhibitor of HCMV and MCMV replication (Shanley and Debs, 1989; Wachsman et al., 1996; Lembo et al., 1998, 1999a,b). TS (E.C.2.1.1.45) is another folate-requiring enzyme recognized as a potential anti-CMV therapeutic target (Wingard et al., 1981; Suzuki et al., 1985, 1987). This enzyme converts deoxyuridine monophosphate (dUMP) into thymidine monophosphate (TMP), and thus catalyzes a key step in cellular and viral DNA synthesis. ZD1694 is a quinazoline-based folate analog TS inhibitor which is approved in Europe for the treatment of cancer (Jackman et al., 1995b; Blackledge, 1998). It is taken up by cells via the reduced folate carrier system and then rapidly converted to polyglutamate forms by folylpolyglutamate synthetase (FPGS) (Jackman and Gibson, 1995). Polyglutamated ZD1694 is a significantly better inhibitor of TS than the parent compound (Jackman et al., 1991). Furthermore, the polyglutamates are retained inside the cells for long time and both enhance and extend the inhibition of TS. The selective activity of ZD1694 against cancer cells is due to its increased polyglutamation and accumulation in actively growing cells as opposed to quiescent cells. The authors and others have demonstrated that CMV efficiently replicates in quiescent cells (Dittmer and Mocarski, 1997; Lembo et al., 1999a), probably through stimulation of pathways normally repressed in these cells, including those involved in dNTP synthesis. The fact that both malignant cells and CMV-infected cells require a large increase in dNTP pools led us to determine whether ZD1694 exerts an anticytomegaloviral activity in quiescent cells.

Here it is reported that ZD1694 potently suppresses the replication of both MCMV and HCMV at concentrations well below those required for its cytotoxic activity. It is also active against two HCMV clinical isolates resistant to GCV due to mutation in the UL97 gene.

2. Materials and methods

2.1. Cells and culture conditions

Low-passage human embryonic lung fibroblasts (PEU) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. NIH 3T3 murine fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum at 37°C in a 5% CO_2 atmosphere.

Quiescent cells (arrested in G_0/G_1 phase) were obtained by incubating confluent monolayers for 72 h in medium containing 0.5% serum (low serum medium).

2.2. Virus preparation and infections

HCMV (strain AD-169, ATCC VR-538) was purchased from the ATCC (Rockville, MD), and stocks were prepared by infecting human embryonic fibroblasts at a virus-to-cell ratio of 0.01. Cells were incubated in MEM supplemented with 1% heat inactivated serum and virus was harvested based on cytopathology at about three weeks post-infection by sonicating, and clarifying by centrifugation.

HCMV VR 5438 and VR 6264 (kindly provided by Professor G. Gerna, Pavia, Italy) are GCV-resistant strains isolated from AIDS patients undergoing GCV therapy: their effective dose 50 (ED_{50}) are 18 μM for VR5438 and 15 μM for VR6264. Previous nucleotide sequence analysis of their UL97 demonstrated amino acid variation of codon 594 (Ala–Val) for VR 6264 and of

codon 595 (Leu–Phe) for VR 5438. These strains were propagated as described for the AD 169 strain.

Mouse salivary gland virus (MCMV, strain Smith, ATCC VR.194) was purchased from the ATCC, and stocks were prepared by infecting Balb/c-mouse embryo fibroblasts (Balb/c-MEF) at a virus-to-cell ratio of 0.01. Cells were incubated in DMEM supplemented with 1% heat inactivated calf serum and virus was harvested based on cytopathology at 7–10 days post-infection by sonicating and clarifying by centrifugation.

Mock-infecting fluids were prepared from uninfected cells in the same way as the virus stocks. Stock solutions containing approximately $1–5 \times 10^6$ plaque forming units (pfu)/ml were used in all experiments. Quiescent cells were infected with HCMV or MCMV at a multiplicity of infection (MOI) of 1, and mock-infected control cultures were exposed to an equal volume of mock-infecting fluid. Virus adsorptions were carried out for 2 h at 37°C, and 0 h post-infection (hpi) is defined as the time immediately following this period.

2.3. Anti-CMV assay

Cells were grown to confluence in 24-well plates, then serum starved and infected with HCMV or MCMV at a multiplicity of infection of 1 pfu/cell (MOI 1). One column per plate was mock-infected and served as cell control. The infected cultures were treated in low serum medium (0.5% calf serum) with different concentrations of ZD1694 (Zeneca) in duplicate wells. In some experiments thymidine was combined with the drug. One column per plate was not treated and served as virus control. Cultures were incubated until the control cultures displayed about 100% cytopathology (usually 96 h for MCMV and 144 h for HCMV). Virus yield was then assessed as described below.

2.4. Virus yield assay

The cells and the supernatants from the anti-CMV assay were harvested and distrupted by sonication. The distrupted cells were centrifuged at $500 \times g$ for 10 min, and the supernatant was assayed for its infectivity by the IE antigen plaque assay for HCMV (Gerna et al., 1992) and by standard plaque assay for MCMV. The number of plaques was plotted as a function of drug concentration and the concentration producing 50% reduction in plaque formation, the effective dose 50 (i.e. 1 ED_{50}), was determined.

2.5. Quantitative PCR

Quantitative PCR for HCMV and MCMV were performed as previously described (Gariglio et al., 1997; Lembo et al., 1999a) In the case of HCMV, the target for PCR amplification was a segment of IE1 exon 4 generating an amplification product of 639 bp. The competitor DNA was a fragment of the same sequence as the target with the sole exception of a 78 bp insertion to enable identification after gel electrophoresis. For MCMV, a DNA fragment of 84 bp was inserted into IE1 exon 3 generating a 723 bp amplification product. Competitive PCR was carried out on 50 ng of sample DNA with the addition of increasing concentrations of the competitor DNA template in 100 μl of PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing the two primers (0.250 μM each), the four dNTPs (200 μM each) and 2.5 U of Taq DNA polymerase (Sigma, St. Louis, MO). Samples were submitted to 30 cycles of amplification with the following cycle profile: denaturation at 94°C for 30 s, annealing to 57°C for 30 s, extension at 72°C for 1 min. Thereafter PCR products were fractionated by 2% agarose gel electrophoresis and the bands corresponding to competitor and genomic DNA were quantified by densitometric scanning.

2.6. Dot blot assay

Inhibition of viral DNA replication was also assayed by dot blot analysis. DNA samples from HCMV and MCMV infected cultures were sequentially hybridized with a ^{32}P-labeled *Pst*I-*Bam*HI DNA fragment containing a portion of the exon 4 of the HCMV IE1 gene, or with a ^{32}P-labeled *Xba*I/*Ava*I DNA fragment containing

a portion of the MCMV IE1 gene, and with a ^{32}P-labeled G3PDH human or murine cDNA. The hybridization signals were quantitated with the Bio-Rad Image Analysis System.

2.7. Cytotoxicity assay

Cells were grown to confluence in 24-well plates and then serum starved. The medium was then replaced by low serum medium (0.5% calf serum) containing increasing concentrations of ZD1694. After 5 days incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988).

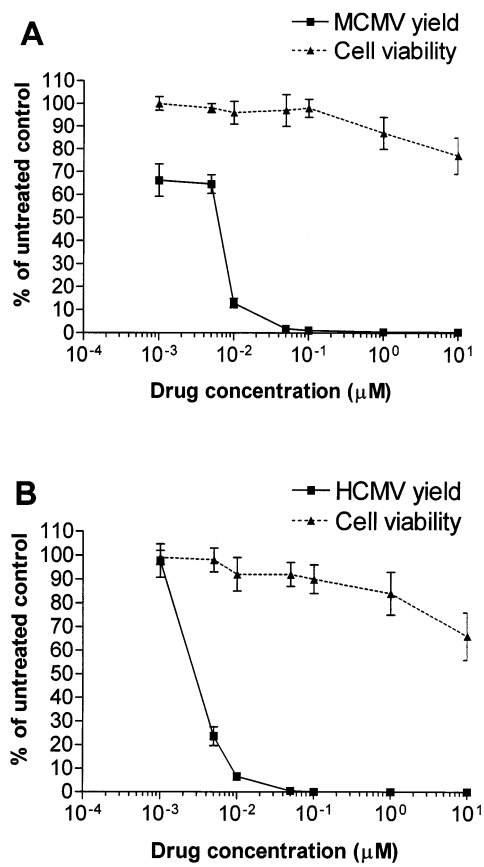

Fig. 1. Inhibitory effect of Tomudex (ZD1694) on murine cytomegalovirus (MCMV) (panel A) and human cytomegalovirus (HCMV) (panel B) replication in quiescent NIH 3T3 and PEU cells. Quiescent cells were infected at a multiplicity of infection (MOI) of 1 and then exposed to increasing ZD1694 concentrations until an extensive viral cytopathic effect was observed in the untreated control. Supernatants of cell suspension were assayed for their infectivity by either standard plaque reduction assay or the IE antigen plaque reduction assay. Values are the means of three separate determinations.

3. Results

3.1. Inhibition of MCMV and HCMV replication by ZD1694

The ability of ZD1694 to inhibit the replication of MCMV and HCMV in murine and human quiescent fibroblasts infected at a MOI of 1 was examined. Fig. 1 shows the results obtained in triplicate assays evaluating virus yield and cell viability. ZD1694 produced a significant dose-related reduction of MCMV (Fig. 1A) and HCMV (Fig. 1B) yield. The calculated 50 and 90% antiviral effective concentrations (EC_{50} and EC_{90}) were 0.006 and 0.01 μM for MCMV and of 0.002 and 0.009 μM for HCMV. ZD1694 did not affect the viability of both human and murine quiescent cells at concentrations up to 1 μM, and its 50% cytotoxic concentration (CC_{50}) was >10 μM, whereas in proliferating human fibroblasts and murine NIH 3T3 cells was of 0.04 and 0.03 μM, respectively. These results indicate that the basis for ZD1694 antiviral activity is not due to cytotoxicity.

To confirm that TS is the sole target of ZD1694 activity under these experimental conditions, thymidine (10 μM) was added in combination with the drug (1 EC_{90}) to the infected cells. Table 1 shows that thymidine completely reversed the antiviral activity of ZD1694.

3.2. Inhibition of MCMV and HCMV DNA synthesis by ZD1694

Viral DNA was quantified by competitive PCR assay to examine the effects of ZD1694 on MCMV and HCMV DNA synthesis. To compare the amount of HCMV DNA in control and ZD1694-treated HCMV-infected PEU cells, total

Table 1
Effect of thymidine on Tomudex (ZD1694) antiviral activity[a]

	MCMV titer (% of untreated control)	HCMV titer (% of untreated control)
1 EC_{90} ZD1694	10	10
+Thymidine (10 μM)	100	89

[a] Quiescent NIH 3T3 and PEU cells were infected with murine and human cytomegalovirus (MCMV and HCMV), respectively (MOI 1) and then exposed to 1 EC_{90} (0.01 μM for MCMV and 0.009 μM for HCMV) of ZD1694, in the absence or with 10 μM thymidine. Supernatants were collected and titrated as described in Section 2.

genomic DNA (50 ng) extracted at 96 hpi was mixed with increasing concentrations of competitor DNA and then subjected to PCR. A set of preliminary experiments was devoted to constructing a standard curve for determination of HCMV DNA in cells infected at an MOI of 1 pfu/cell. The ratio between competitor and target amplification products was found linear over a competitor input ranging from from 5×10^6 to 5×10^9 molecules ($R^2 = 0.988$). As shown in Fig. 2B, equivalence between target and competitor corresponding to a 1:1 ratio and therefore indicating the number of molecules of target initially present in the reaction, was obtained for control DNA at a concentration of about 5×10^8 molecules, whereas for DNA extracted from ZD1694-treated cells (0.1 μM) the equivalence between the two PCR products was reached at about 5×10^7 molecules. These results demonstrated that ZD1694 inhibited viral DNA synthesis about 10-fold. A similar degree of inhibition was calculated for MCMV (Fig. 2A).

Finally, to determine whether inhibition included other events in the HCMV growth cycle, the expression of immediate-early 1 (IE1) and a late antigen (LA) was examined by standard immunofluorescence analysis. LA, but not IE1 expression was inhibited by 1 ED_{90} ZD1694 (data not shown).

3.3. Reversibility of ZD1694 treatment

A dot blot assay was then conducted to determine the duration of ZD1694 exposure required to inhibit MCMV and HCMV replication. Quies-

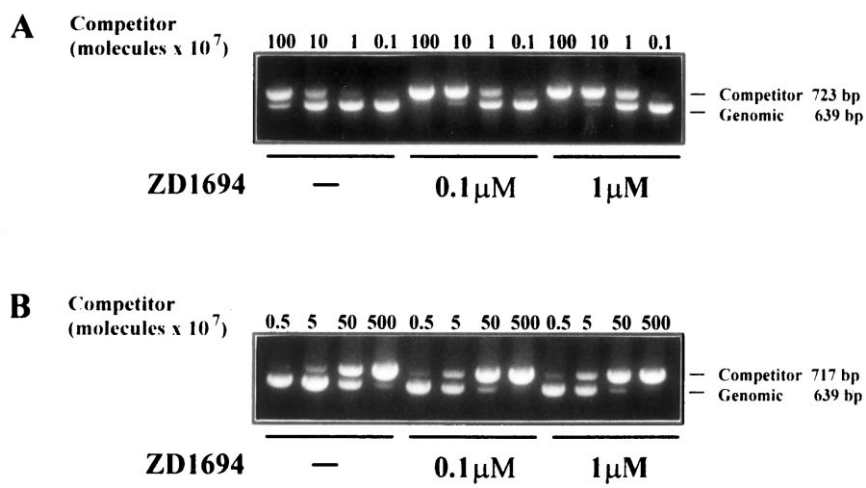

Fig. 2. Inhibitory effect of Tomudex (ZD1694) on murine cytomegalovirus (MCMV) (panel A) and human cytomegalovirus (HCMV) (panel B) DNA synthesis in quiescent NIH 3T3 and PEU cells Cells were infected at a multiplicity of infection (MOI) of 1 and exposed to increasing ZD1694 concentrations. Total genomic DNA was then extracted at 48 (MCMV) and 96 h post-infection (hpi) (HCMV), purified and mixed with increasing concentrations of competitor DNA ranging from 10^6 to 10^9 molecules, and subjected to PCR. PCR products were then fractionated on a 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The positions of genomic and competitor DNA are indicated on the right.

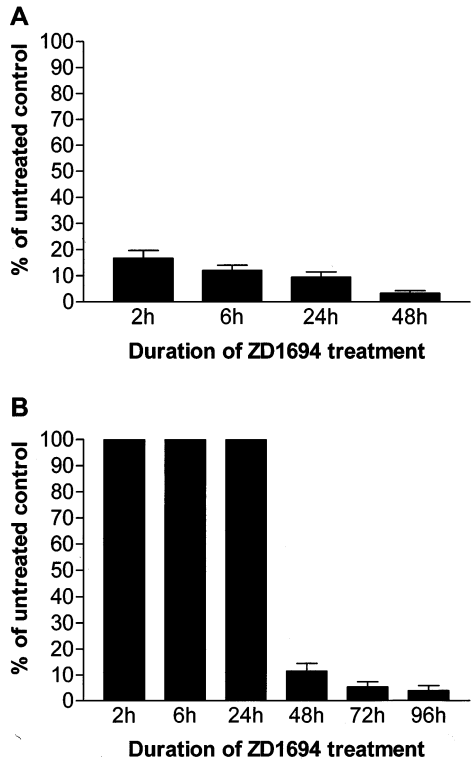

Fig. 3. Effect of duration of Tomudex (ZD1694) exposure on inhibition of murine cytomegalovirus (MCMV) (panel A) and human cytomegalovirus (HCMV) (panel B) DNA synthesis in quiescent NIH 3T3 and PEU cells. Cells were infected at a multiplicity of infection (MOI) of 1 and then exposed to 0.1 μM of ZD1694 for the indicated times. Thereafter, the drug was removed, total genomic DNA was extracted at 48 hpi (MCMV) and 96 h post-infection (hpi) (HCMV) and immobilized on a hybridization membrane by a dot blot apparatus. The filters were sequentially hybridized with ^{32}P-labeled viral probes (fragments from both the MCMV and HCMV IE1 genes) and with cellular DNA probes (full length G3PDH cDNAs). The hybridization signals were quantitated with the Bio-Rad Image Analysis System and adjusted to the differences in G3PDH gene levels.

cent fibroblasts were infected with MCMV and HCMV (MOI 1), and ZD1694 (0.1 μM) was added immediately after virus adsorption. Drug was then removed at indicated times by three 30-min washes with drug-free medium at 37°C. All MCMV- and HCMV-infected cultures were collected at 48 and 96 hpi, respectively, and DNA was extracted. As shown in Fig. 3A, the presence of ZD1694 for just the first two hours inhibited MCMV replication by more than 80%, whereas it had no effect on HCMV replication during the first 24 h. However, if the drug was removed 48 h after infection, HCMV DNA synthesis did not resume and was greatly suppressed (Fig. 3B).

3.4. ZD1694 inhibits the replication of GCV-resistant HCMV strains

Isolation of GCV-resistant HCMV strains has been frequently reported during prolonged treatment of AIDS patients. Mutations in both UL97 and DNA polymerase genes may induce this resistance (1). To determine whether ZD1694 is effective in this context, its efficacy against two GCV-resistant strains deficient in drug phosphorylation due to mutations in the UL97 gene is measured. A quantitative PCR analysis (Fig. 4) demonstrated that they were sensitive to ZD1694 at a level comparable to that obtained for the AD 169 strain.

4. Discussion

Cytomegalovirus replication in vivo occurs in terminally differentiated cells of epithelial and endothelial origin that are actively inhibited to enter the cell cycle. In this study, quiescent fibroblasts were used as an in vitro experimental system of post-mitotic cells. The results show that ZD1694 is potent against both MCMV and HCMV and inhibits their replication by inhibiting their DNA synthesis and late gene expression. Moreover the finding that IE gene expression was not suppressed under conditions where viral DNA synthesis is inhibited demonstrates that early events in the CMV replication cycle are not affected by ZD1694 treatment. It was also found that TS is its sole target, since its effects are reversed by thymidine alone. This finding demonstrates that the ZD1694 antiviral activity is specific and does not depend on a generalized disfunction of host cell metabolism provoked by drug concentrations below those affecting cell viability.

ZD1694 inhibits TS by binding at its folate rather than at its pyrimidine-binding site. This

binding inhibits DNA synthesis and repair by blocking the obligatory thymidine nucleotide synthesis. Like other analogs of folate cofactors, the drug's biological activity depends upon active uptake via the reduced folate/MTX cell membrane carrier and subsequent metabolism by FPGS to polyglutamated forms (tri, tetra and pentaglutamates). These polyglutamates are approximately 100-fold more active as TS inhibitors and are not effluxed (Jackman et al., 1991; Fleming and Schilsky, 1992). ZD1694 has proved active in Phase I, II and III clinical trials against ovarian, lung, colon and gastric carcinomas (Jackman et al., 1995b; Blackledge, 1998). This is the first report of its antiviral activity. Its potent inhibitory action against CMV in quiescent cells deserves some comments.

First, the ZD1694 selectivity may depend on the very low levels of TS and FPGS in quiescent uninfected cells since the expression of both enzymes is cell-cycle-regulated and only significantly high in proliferating cells (Navalgund et al., 1980; Barredo and Moran, 1992; Johnson, 1994). Second, it has recently been observed that CMV infection induces both FPGS and TS expression at the mRNA and the protein level (unpublished results). The selective induction of FPGS and TS in CMV-infected cells, could provide a specific target for anti-CMV drugs. It follows that, in quiescent cells, inhibition of TS by ZD1694 may be deleterious only to the rapidly replicating viral DNA.

Third, the use of small organic molecules as inhibitors of cellular functions may provide important informations on the role of these cellular process in the virus replication cycle. In fact, inhibition of viral replication by ZD1694 and its abrogation by 10 μM thymidine clearly demonstrate that TS activity is required for efficient CMV replication in quiescent cells. These findings, along with those previously reported by us showing the increase of DHFR activity upon CMV infection (Lembo et al., 1998, 1999a), demonstrate that virus infection of quiescent cell depends on the activation of cellular enzymes involved in the synthesis of dTMP.

Flourinated pyrimidine-based TS inhibitors have been examined for inhibition of CMV replication (Suzuki et al., 1985, 1987). However, they need to be delivered into the cell as a base and have to be phosphorylated by cellular TK, since CMV does not express TK, to the active species. In addition, fluorinated pyrimidines are extensively metabolized intracellularly to several anabolites other than FdUMP, so that incorporation into RNA and DNA may occur. Furthermore the large expansion in the levels of the competing substrate, dUMP, that occurs fol-

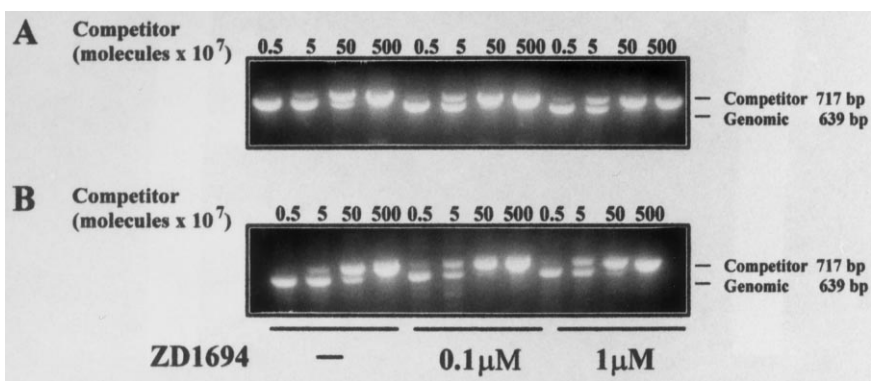

Fig. 4. Inhibitory effect of Tomudex (ZD1694) on GCV-resistant human cytomegalovirus (HCMV) VR5438 (panel A) and VR6264 (panel B) strains in quiescent PEU cells. Cells were infected at a MOI of 1 and then exposed to increasing ZD1694 concentrations. Total genomic DNA was then extracted at 144 h post-infection (hpi), purified and mixed with increasing concentrations of competitor DNA ranging from 10^6 to 10^9 molecules and subjected to PCR. PCR products were then fractionated on a 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The positions of genomic and competitor DNA are indicated on the right.

lowing TS inhibition by FdUMP may impair the optimal enzyme inactivation (Jackman et al., 1985). These additional and secondary effects have been overcome by the design of analogs of the folate cofactor rather than the pyrimidine substrate. A number of promising new antifolates that target TS have entered in clinical oncology trials in recent years. ZD1694, for example has proved more effective than either MTX or 5-Fu against a panel of ten human tumor xenografts (Jackman et al., 1995b).

MTX exerts a potent anti-CMV activity and preferentially accumulates in infected cells, where it is found almost entirely in the polyglutamated form, rather than uninfected cells, where it is almost exclusively in the parent form (Wachsman et al., 1996). A similar mechanism can be envisaged for CMV-infected and ZD1694-treated cells. Here, selective activation of FPGS would increase TS inhibtion by ZD1694, because, unlike MTX, its polyglutamated forms inhibit the target enzyme to a greater extent than the parent drug.

In this study, the antiviral activity of ZD1694 was irreversible and CMV DNA synthesis did not resume after removal of the drug. Two mechanisms may explain this phenomenon. First, it has been demonstrated that cellular retention of polyglutamated ZD1694 results in prolonged TS inhibition after the extracellular drug is removed (Jackman et al., 1991; Jackman and Gibson, 1995). Second, the ability of ZD1694 to induce both mature and nascent DNA fragmentation may be an important additional determinant of its irreversible effects on CMV replication (Yin et al., 1992; Panadero et al., 1995).

GCV is the anti-HCMV drug most widely used in clinical practice. However, its inhibitory effects are reversible, since DNA synthesis and replication resume when it is removed (Mar et al., 1982, 1983). Prolonged treatment leads to the emergence of resistant strains, particularly when GCV is employed to manage disseminated infections in AIDS patients. The irreversible antiviral activity of ZD1694 may lead to lengthy suppression after it is removed and permit a more convenient dosing schedule than is possible for regimens based on virustatic drugs, and the results suggest that it might be used to manage infections by GCV-resistant strains. Combination of 3′-azido-3′-deoxythymidine (AZT) with ZD1694 has shown that AZT increases the antitumor effect of ZD1694 with increasing AZT incorporation into DNA and minimal myelosuppression (Pressacco and Erlichman, 1993). The studies suggest that this combination warrants further investigation as an anti-HCMV and HIV regimen.

In conclusion this work indicates that suppression of TS activity by polyglutamable drugs may represent a strategy to inhibit CMV replication. When ZD1694 entered Phase I evaluation a maximum tolerated dose of 3.5 mg/m^2 (given once every 3 weeks) was established. The most frequent toxicities at this dose included gastrointestinal (approximately 20%) and hematological (mainly neutropenia, approximately 23%) (Jackman et al., 1995b). All the anti-CMV assays presented in this paper were performed in serum-starved cells as an in vitro model of post-mitotic cells which represent the most likely target of CMV infection in vivo. The authors are aware that ZD1694 is endowed of a cytotoxic activity against replicating cells. In fact, it has been observed that in proliferating human fibroblasts its CC_{50} was of 0.04 μM, whereas a value 0.025 μM has been reported for human bone marrow CFU-GM cells (Pressacco and Erlichman, 1993). In quiescent cells, the ZD1694 EC_{50} against HCMV is 0.002 μM (Fig. 1), 20-fold lower than the CC_{50} in replicating fibroblasts. Whether this difference can be exploited to set up an antiviral regimen in vivo remains to be established. Therefore, an animal model is required to evaluate if ZD1694 exhibits antiviral activity in vivo at drug concentrations below those inducing toxicity and to derive a therapeutic index. The authors plan to study the anticytomegaloviral activity of ZD1694 in SCID mice infected with MCMV as a model of HCMV infection in immunocompromised individuals. These studies will establish the potential of ZD1694 to control HCMV infections in humans.

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