Inhibitors of human cytomegalovirus replication drastically reduce the activity of the viral protein kinase pUL97

Manfred Marschall,1 Matthias Stein-Gerlach,2 Martina Freitag,1 Regina Kupfer,1 Miriam van den Bogaard2 and Thomas Stamminger1

1 Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Schloßgarten 4, 91054 Erlangen, Germany
2 Axxima Pharmaceuticals AG, Martinsried, Germany

The UL97-encoded protein kinase (pUL97) of human cytomegalovirus (HCMV) plays a critical role in the control of virus replication. Deletion of the UL97 gene results in a drastic reduction in the replication efficiency. Although the exact function of pUL97 remains unclear and its sensitivity to specific inhibitors is speculative, protein kinase inhibitors of the indolocarbazole class are effective inhibitors of cytomegalovirus. Based on the phosphorylation of ganciclovir (GCV), a novel quantification system for pUL97 kinase activity was established: the phosphorylated form of GCV exerts an easily quantifiable cytotoxic effect in transfected cells. Importantly, the addition of indolocarbazole compounds, Gö6976 and NGIC-I, which were highly effective at nanomolar concentrations while other protein kinase inhibitors were not, led to a significant reduction of pUL97 kinase activity. It was also demonstrated that a catalytically inactive mutant of pUL97, K355M, and a GCV-resistant mutant, M460I, were both negative for GCV phosphorylation, although protein phosphorylation remained detectable for the latter mutant. In vitro kinase assays were used to confirm the levels of pUL97-mediated phosphorylation recorded. To generate a tool for screening large numbers of putative inhibitors that preferentially interfere with GCV as well as protein phosphorylation, pUL97-expressing cell clones with stable pUL97 kinase activity were selected. This study demonstrates that certain indolocarbazole compounds are potent pUL97 inhibitors and, therefore, represent novel candidates for antiviral drugs that target viral protein kinase functions.

Introduction

Human cytomegalovirus (HCMV) is a major pathogen that induces a variety of diseases in high-risk individuals. Immunosuppression, for example, often triggers the reactivation of a persistent HCMV infection causing the onset of severe symptoms, such as retinitis, pneumonitis and gastroenteritis. Other immunocompromised individuals, such as organ transplant recipients, frequently suffer from systemic HCMV infection, and congenital infection in the context of severe, generalized cytomegalic inclusion disease is highly problematic (reviewed by Britt & Alford, 1996).

To date, therapeutic antiviral compounds against HCMV are limited. Ganciclovir (GCV), cidofovir and foscarin inhibit HCMV genome replication, either directly or indirectly, but these drugs may induce the formation of resistant virus, have low oral bioavailability and show dose-related toxicity (reviewed by Hayden, 1995). However, the panel of anti-HCMV compounds becoming available is expanding. Firstly, the phosphorothioate oligonucleotide fomivirsen (ISIS 2922) is a sequence-specific inhibitor of transcription of the major immediate early gene region which effectively blocks the onset of the virus replication cycle (reviewed by Perry & Balfour, 1999). Secondly, benzimidazole riboside compounds (BDCRB, TCRB, 1263W94 and others) possess inhibitory capacities towards different stages of HCMV replication (for example, genomic DNA maturation) and represent attractive candidates for orally applicable and low-toxicity therapeutic compounds (reviewed by Chulay et al., 1999). Thirdly, a novel compound, termed A771726 [N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotoamide], which is the active metabolite of the anti-
inflammatory drug leflunomide, has recently been described as a potent inhibitor of HCMV replication, probably acting at the late stage of infection by preventing virion assembly (Waldman et al., 1999a, b). The general value of most of these compounds for use as antiviral drugs will have to be proven through clinical trials.

pUL97 is a well-characterized target for antiviral therapy, as the specific activity of pUL97 is required to convert GCV into its monophosphorylated derivative (Littler et al., 1992; Sullivan et al., 1992). Cellular enzymes further phosphorylate GCV into its triphosphated form, which inhibits viral DNA synthesis by inhibiting the viral DNA polymerase (reviewed by Hayden, 1995). Several reports have shown that pUL97 acts as a serine/threonine-specific protein kinase that can phosphorylate itself and other proteins (Chee et al., 1989; He et al., 1997; Kawaguchi et al., 1999; Michel et al., 1998, 1999; van Zeijl et al., 1997; Wolf et al., 1998). Interestingly, functional domains for protein and GCV phosphorylation are partially distinct (Michel et al., 1998; Wolf et al., 1998) and natural nucleosides are not recognized substrates of the pUL97 kinase, indicating that nucleoside kinase activity is not its principal function (Michel et al., 1996). A recent publication by Slater et al. (1999) provided the first evidence that protein kinase inhibitors are potent and selective antagonists of HCMV replication in tissue culture and as pUL97 function is critical for efficient HCMV replication (Michel et al., 1996; Prichard et al., 1999), it was considered to be a promising target for antiviral therapy.

In the present work, we established a novel system for examining pUL97 kinase activity in transfected cells. By quantifying the cytotoxic effects induced by the conversion of GCV into its phosphorylated derivatives (Chee et al., 1989; He et al., 1997; Kawaguchi et al., 1999; Michel et al., 1998, 1999; van Zeijl et al., 1997; Wolf et al., 1998), we determined the activity of pUL97. Furthermore, additional information about the kinase activity and characteristics of pUL97 mutants was obtained. A screening system for antiviral agents is also presented.

Methods

**Plasmid constructs.** The UL97 ORF of HCMV strain AD169 was amplified by PCR (Vent DNA polymerase, New England BioLabs; 35 cycles each comprising 40 s at 95 °C for denaturation, 40 s at 50 °C for annealing and 120 s at 72 °C for polymerization). After digestion with the respective restriction enzymes, the UL97 ORF was used to produce the following plasmid constructs (Table 1):

1. pcDNA-UL97 and pcDNA-UL97(M460I) – PCR primers 1 and 4 were used with pcDNA3 (Invitrogen) [ORF-UL97(M460I) was derived from the GCV-resistant virus mutant AD169–GFP314 (Marschall et al., 2000)].
2. pcDNA-UL97(K355M), pcDNA-UL97–FLAG, pcDNA-UL97–VSV and pcDNA-UL97–HA – PCR primers 2 and 5, 6, 7 or 8, respectively, were used with pcDNA3 [ORF-UL97(K355M) was generated by site-directed mutagenesis (Kunkel, 1985)]. The primers describe the wild-type UL97 ORF and the mutagenesis primer 9 were used to substitute the codon AAG (lysine) with ATG (methionine).
3. pSC-UL97 and p18neo-UL97 – PCR primers 1 and 4 were used with either pSuperCatch (Georgiev et al., 1996) or p18neo (Marschall et al., 1999).
4. pLXS-N-UL97 and pCmn-UL97 – PCR primers 2 or 3, respectively, and 5 were used with either pLXSN (Clontech) or pCMV/myc/nuc (Invitrogen). pCmn-GFP is a green fluorescent protein (GFP)-encoding expression construct of the pCMV/myc/nuc vector used as a standard transfection control.

**Oligonucleotides.** Synthetic oligonucleotide primers for PCR were purchased from Sigma. HCMV-specific sequences are underlined. Restriction sites are indicated in bold and tagged or mutated sequences are indicated in italics. The sequences of the forward primers used are as follows:

1. 5-UL97-BglII (TAGTAGATCTATGTTCGCCACCTTCGGTTCT)
2. 5-UL97-EcoRI (CCCCGATTTCATGTCTCCGACCTT)
3. 5-UL97-Ncol (CATGCCATGGCATCTCCGACCTT)
4. 3-UL97-Sall (TAGTGTCCGACATCTCCGGGAAAGCTTT)
5. 3-UL97-XhoI (CCCTCGAGTTACTCGGGGAACAGTTGGTCTTGTGTAGTGTCTCGGGGAACAGTTG)
6. 3-UL97-FLAG-XhoI (CCCTCGAGTTACTGTCTCGGGGAACAGTTG)
7. 3-UL97-VSV-XhoI (CCCTCGAGTTACTGGCCACCGGGTGCTATCTGCGATCTCGGGGAACAGTTG)
8. 3-UL97-314-HA-XhoI (CCCGTCGAGTTACGGGTAACGGTGTCTAGCGGGGAACTTTTTTCTCGGGGAACAGTTG)
9. Mut355-UL97 (CTTACGCCACCATACGACCGGATATATGTTTTGCTAGT)

**Protein kinase inhibitors.** Staurosporine (STP), G6976, G67874 and NGIC-1 are inhibitors of serine/threonine protein kinases. AG-490 (tyrphostin B42) is an inhibitor of tyrosine protein kinases (Meydan et al., 1996). All compounds were purchased from Calbiochem. Stock solutions were prepared in DMSO and stored at −20 °C.

**Cultured cells and infection procedures.** 293 cells (human embryonic kidney cells) were cultivated in Dulbecco’s minimal essential medium (DMEM) containing 5% foetal calf serum (FCS) and 100 µg/ml gentamycin. Primary human foreskin fibroblast (HFF) cell cultures were grown in minimal essential medium (MEM) containing 5% FCS and 100 µg/ml gentamycin. Subconfluent monolayers were used for HCMV strain AD169 infection. 

---

**Methods**

**Plasmid constructs.** The UL97 ORF of HCMV strain AD169 was amplified by PCR (Vent DNA polymerase, New England BioLabs; 35 cycles each comprising 40 s at 95 °C for denaturation, 40 s at 50 °C for annealing and 120 s at 72 °C for polymerization). After digestion with the respective restriction enzymes, the UL97 ORF was used to produce the following plasmid constructs (Table 1):

1. pcDNA-UL97 and pcDNA-UL97(M460I) – PCR primers 1 and 4 were used with pcDNA3 (Invitrogen) [ORF-UL97(M460I) was derived from the GCV-resistant virus mutant AD169–GFP314 (Marschall et al., 2000)].
2. pcDNA-UL97(K355M), pcDNA-UL97–FLAG, pcDNA-UL97–VSV and pcDNA-UL97–HA – PCR primers 2 and 5, 6, 7 or 8, respectively, were used with pcDNA3 [ORF-UL97(K355M) was generated by site-directed mutagenesis (Kunkel, 1985)]. The primers describe the wild-type UL97 ORF and the mutagenesis primer 9 were used to substitute the codon AAG (lysine) with ATG (methionine).
3. pSC-UL97 and p18neo-UL97 – PCR primers 1 and 4 were used with either pSuperCatch (Georgiev et al., 1996) or p18neo (Marschall et al., 1999).
4. pLXS-N-UL97 and pCmn-UL97 – PCR primers 2 or 3, respectively, and 5 were used with either pLXSN (Clontech) or pCMV/myc/nuc (Invitrogen). pCmn-GFP is a green fluorescent protein (GFP)-encoding expression construct of the pCMV/myc/nuc vector used as a standard transfection control.

**Oligonucleotides.** Synthetic oligonucleotide primers for PCR were purchased from Sigma. HCMV-specific sequences are underlined. Restriction sites are indicated in bold and tagged or mutated sequences are indicated in italics. The sequences of the forward primers used are as follows:

1. 5-UL97-BglII (TAGTAGATCTATGTTCGCCACCTTCGGTTCT)
2. 5-UL97-EcoRI (CCCCGATTTCATGTCTCCGACCTT)
3. 5-UL97-Ncol (CATGCCATGGCATCTCCGACCTT)
4. 3-UL97-Sall (TAGTGTCCGACATCTCCGGGAAAGCTTTTTTCTCGGGGAACAGTTG)
5. 3-UL97-XhoI (CCCTCGAGTTACTCGGGGAACAGTTGGTCTTGTGTAGTGTCTCGGGGAACAGTTG)
6. 3-UL97-FLAG-XhoI (CCCTCGAGTTACTGTCTCGGGGAACAGTTG)
7. 3-UL97-VSV-XhoI (CCCTCGAGTTACTGGCCACCGGGTGCTATCTGCGATCTCGGGGAACAGTTG)
8. 3-UL97-314-HA-XhoI (CCCGTCGAGTTACGGGTAACGGTGTCTAGCGGGGAACTTTTTTCTCGGGGAACAGTTG)
9. Mut355-UL97 (CTTACGCCACCATACGACCGGATATATGTTTTGCTAGT)

**Protein kinase inhibitors.** Staurosporine (STP), G6976, G67874 and NGIC-1 are inhibitors of serine/threonine protein kinases. AG-490 (tyrphostin B42) is an inhibitor of tyrosine protein kinases (Meydan et al., 1996). All compounds were purchased from Calbiochem. Stock solutions were prepared in DMSO and stored at −20 °C.

**Cultured cells and infection procedures.** 293 cells (human embryonic kidney cells) were cultivated in Dulbecco’s minimal essential medium (DMEM) containing 5% foetal calf serum (FCS) and 100 µg/ml gentamycin. Primary human foreskin fibroblast (HFF) cell cultures were grown in minimal essential medium (MEM) containing 5% FCS and 100 µg/ml gentamycin. Subconfluent monolayers were used for HCMV strain AD169 infection.
The UL97 ‘in-cell-activity’ assay. On day 0, 293 cells were seeded in 96-well plates (20,000 per well) to obtain 50% confluent monolayers. On day 1, transfection was performed according to the lipofectamine procedure (Lipofectamine Plus reagents, Gibco BRL). For this, identical transfection conditions were chosen for 24 wells of the 96-well plate so that measurements over an 8-well line could be carried out in triplicate. Components A [2.5–10 µg plasmid DNA (UL97 expression construct or control), 300 µl FCS-free DMEM and 25 µl Plus reagent] and B (12.5 µl Lipofectamine reagent and 300 µl FCS-free DMEM) were used for each transfection assay. Both components were incubated for 15 min at room temperature. Then, A and B were combined, mixed thoroughly and incubated for 15 min at room temperature. Meanwhile, the culture media of the 96-well plate were removed with a multichannel pipette and replaced by 50 µl of fresh FCS-free DMEM per well. An aliquot of 25 µl of each transfection mixture (AB) was added per well. Plates were incubated for 5 h at 37 °C in 5% CO₂. Subsequently, 125 µl DMEM supplemented with 10% FCS was added per well and incubated overnight at 37 °C in 5% CO₂. On day 2, transfection media were removed from the cells. GCV was diluted in DMEM containing 5% FCS to create a gradient of appropriate GCV concentrations and added in a volume of 100 µl per well. Protein kinase inhibitors were diluted in DMEM containing 5% FCS and added in a volume of 100 µl per well immediately after the addition of GCV. Plates were incubated at 37 °C in 5% CO₂.

Visual evaluation of transfected cells and quantification of the colour change in the culture media. On day 7, visual evaluation of plates became possible by the colour change (yellow to red) in the culture media. Visual evaluation of transfected cells and quantification of cytotoxic effects under a microscope was confirmed by examining cytotoxic effects under a microscope and photometric quantification of the colour change was achieved using an ELISA plate reader (OD₅₆₀).

Quantification of cytotoxicity signals from cell layers. Cytotoxicity signals were quantified in the residual cell layers using the Cytotox 96 nonradioactive cytotoxicity assay kit (Promega), which measures lactate dehydrogenase (LDH) activity in cell lysates 5 days post-transfection. For this, culture media were removed, cells were rinsed with PBS and lysed in a 1 × concentration of the kit lysis buffer (100 µl per well). After 45 min of incubation at 37 °C, cell debris was removed by centrifugation and 5 µl of each lysate was diluted in a total of 50 µl PBS to determine LDH activity. A sample of 50 µl of substrate mix was added to each well and incubated for 30 min at room temperature in the dark. Thereafter, 50 µl of stop buffer was added and the colour reaction was quantified using an ELISA plate reader (OD₅₆₀).

Double-selection protocol for UL97-expressing cell clones. 293 cells were transfected with either pUL97 expression constructs or control plasmids encoding a geneticin-selectable marker and selected for geneticin resistance (750 µg/ml). Individual clones were isolated and selected (in parallel) for either geneticin resistance alone (cell stock plate) or for geneticin resistance in addition to the ability to convert GCV (100 µM) (activity test plate). Clones expressing active pUL97 kinase were cultivated from the cell stock plate and used for large-scale screening of compounds that inhibit pUL97 kinase activity.

UL97 in vitro kinase assay. 293 cells were seeded in 12-well plates in a volume of 1 ml (20,000 cells per well) 24 h before transfection. Transfection was performed according to the lipofectamine procedure described above and cells were incubated for 16 h at 37 °C and 3% CO₂. Cells were washed with PBS and fresh medium was added. Cells were then cultured for a further 24 h. Indolocarbazole compounds were added to the culture media 2 h before lysis. As a control, DMSO was added to monitor the effect of the solvent. Finally, media were removed and cells were lysed in 200 µl of lysis buffer for 30 min (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Triton X-100, 1 mM sodium deoxycholate, 0.1% SDS, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM PMSF and 10 µg/ml aprotinin). After lysis, samples were centrifuged at 4 °C for 30 min at 13,000 r.p.m. Supernatants were transferred to fresh tubes and used for immunoprecipitation with UL97 antiserum (PepAs 1343; 1:5 µl/supernatant) together with 60 µl of a solution of protein A–Sepharose beads (Pharmacia) and an additional 500 µl of lysis buffer. After 2.5 h of incubation at 4 °C on an overhead rotary wheel, precipitates were centrifuged at 2000 r.p.m. at 4 °C for 2 min in an Eppendorf centrifuge and washed twice with 500 µl of HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine and 0.1% Triton X-100). For the in vitro kinase assay, washing was repeated with 500 µl of kinase base buffer (50 mM ChEs, pH 9.5, 10 mM MgCl₂ and 1 mM sodium orthovanadate) and, thereafter, samples were incubated with 40 µl of complete kinase buffer for 30 min on a shaker at 30 °C (kinase base buffer, 2 mM DTT, 2 mM ATP, 1 µCi/sample

### Table 1. Attributes of plasmid constructs

<table>
<thead>
<tr>
<th>Designation</th>
<th>Vector</th>
<th>Inserted gene</th>
<th>Fused tag</th>
<th>Mutation</th>
<th>Expressed phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA-UL97</td>
<td>pcDNA3</td>
<td>UL97 (1–707)</td>
<td>–</td>
<td>–</td>
<td>Kinase activity</td>
</tr>
<tr>
<td>pcDNA-UL97(K355M)</td>
<td>pcDNA3</td>
<td>UL97 (1–707)</td>
<td>–</td>
<td>K355M</td>
<td>Catalytically inactive mutant</td>
</tr>
<tr>
<td>pcDNA-UL97(M460I)</td>
<td>pcDNA3</td>
<td>UL97 (1–707)</td>
<td>–</td>
<td>M460I</td>
<td>GCV-resistant mutant</td>
</tr>
<tr>
<td>pcDNA-UL97–FLAG</td>
<td>pcDNA3</td>
<td>UL97 (1–707)</td>
<td>FLAG</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>pcDNA-UL97–SVS</td>
<td>pcDNA3</td>
<td>UL97 (1–707)</td>
<td>VSV G</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>pcDNA-UL97–HA</td>
<td>pcDNA3</td>
<td>UL97 (1–707)</td>
<td>HA</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>p5SC-UL97</td>
<td>pSuperCatch</td>
<td>UL97 (1–707)</td>
<td>FLAG</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>p18neo-UL97</td>
<td>p18neo</td>
<td>UL97 (1–707)</td>
<td>–</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>pLSXN-UL97</td>
<td>pLSXN</td>
<td>UL97 (1–707)</td>
<td>–</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>pCmn-UL97</td>
<td>pcCMV/myc/nuc</td>
<td>UL97 (1–707)</td>
<td>c-myc, NLS</td>
<td></td>
<td>Kinase activity</td>
</tr>
<tr>
<td>pCmn–GFP</td>
<td>pcCMV/myc/nuc</td>
<td>gfp (1–238)</td>
<td>c-myc, NLS</td>
<td></td>
<td>GFP expression</td>
</tr>
</tbody>
</table>
Quantification of pUL97 kinase activity

HCMV pUL97 was expressed in transiently transfected 293 cells. To quantify its kinase activity, a novel method, termed the UL97 in-cell-activity assay, was developed (Fig. 1).

Results

Quantification of pUL97 kinase activity

HCMV pUL97 was expressed in transiently transfected 293 cells. To quantify its kinase activity, a novel method, termed the UL97 in-cell-activity assay, was developed (Fig. 1).

The expression of recombinant pUL97 results in the in-tracellular phosphorylation of GCV, which induces apoptosis and cytotoxic effects. Thus, pUL97 kinase activity can be quantified in UL97-transfected cells by measuring the extent of this cytotoxicity. Quantification was performed either by visual evaluation of the assay plates or by photometric determination of the colour change in the culture media. Due to the fact that the Phenol Red-related colour change might be influenced by chemical features of the tested drugs or other external factors that alter the pH, alternative quantification methods, such as the LDH activity-based cytotoxicity assay, were established.

Firstly, different plasmid constructs expressing authentic versions of pUL97 (pI6neo-UL97 and pcDNA-UL97) and tagged fusions of pUL97 (pSC-UL97, pcDNA-UL97-FLAG, pcDNA-UL97-βVSV and pcDNA-UL97-HA) were transfected into 293 cells (Fig. 2A). All constructs encoding intact pUL97 (authentic and tagged versions) were effective at inducing into 293 cells (Fig. 2A). All constructs encoding intact pUL97 (pSC-UL97, pcDNA-UL97–FLAG, pcDNA-UL97–βVSV and pcDNA-UL97–HA) were transfected into 293 cells (Fig. 2A). All constructs encoding intact pUL97 (authentic and tagged versions) were effective at inducing.

Indolocarbazole compounds drastically reduce pUL97 kinase activity

Indolocarbazole compounds (derivatives of the lead compound STP), which have been recently characterized as inhibitors of HCMV replication, were analysed for their inhibitory effects on the viral protein kinase pUL97. It should be stressed that for STP and its derivatives NGIC-I, G6976 and G07874, in particular, the inhibitory effect on serine/threonine-specific kinases of the PKC family has been described in several publications (Hu et al., 1996; Pindur et al., 1999; reviewed by Goekjian & Jirousek, 1999), but to date, their effect on herpesvirus protein kinases remains in question. In the UL97 in-cell-activity assay, we identified a clear reduction in pUL97 activity (as measured by the level of GCV phosphorylation) following treatment with either NGIC-I or G6976. In contrast, no change in pUL97 activity was noted for another indolocarbazole compound, G07874, or for the compound AG-490, a tyrosine kinase-specific inhibitor that was added for comparison (Fig. 3A). In addition to these examples, a large number of other indolocarbazole compounds were tested in the UL97 in-cell-activity assay. Taken together, the results confirmed the strong inhibitory activity of NGIC-I.
Inhibitors of HCMV pUL97 kinase activity

Fig. 1. Principle of the UL97 in-cell-activity assay. Active pUL97 kinase is constitutively expressed after plasmid transfection into cultured cells (HCMV IE-pe, immediate early promoter enhancer). Addition of GCV leads to its pUL97-mediated conversion into GCV monophosphate, which exerts a strong cytotoxic effect. Cytotoxicity can be quantified by different means as indicated (a)–(c). Inhibitors of pUL97 kinase activity are able to block this effect. A catalytically inactive mutant, pUL97 (K355M), is used as a control. Note, however, that in the case of the active pUL97 kinase (*), the reduction in cell death indicates the high specificity of the inhibitor, and that in the case of the catalytically inactive mutant of pUL97 (**), the increase in cell death indicates the inherent cytotoxicity of the inhibitor.

Different kinase activities and inhibitor sensitivities of pUL97 mutants

Two mutants of pUL97 were analysed for their activity in phosphorylating GCV or protein: the catalytically inactive mutant K355M and the mutant M460I derived from a GCV-resistant variant of HCMV (Marschall et al., 2000) known to confer GCV resistance (Erice, 1999). As shown by Western blot analysis, wild-type pUL97 and the two point mutants of pUL97 were expressed in transfected cells (Fig. 4A). In the UL97 in-cell-activity assay (Fig. 4 B–D), only wild-type pUL97 kinase showed high-level activity, while both mutants clearly showed a defect in converting GCV (5–40 µM). In the case of the wild-type protein, addition of NGIC-I led to a drastic inhibition of pUL97 kinase activity in a concentration-dependent manner (Fig. 4 B). In comparison, addition of NGIC-I to the kinase mutants K355M and M460I did not result in a change of kinase activity. Interestingly, the addition of STP to both mutants caused nonspecific cytotoxic effects (Fig. 4 C, D). Comparison of these panels shows that the nonspecific cytotoxicity of STP can be distinguished from the specific pUL97-inhibiting effect of NGIC-I, which is only detectable for wild-type pUL97. When examined for protein phosphorylation, however, the two pUL97 mutants showed individual characteristics. While mutant K355M was com-
Fig. 2. Establishment of the UL97 in-cell-activity assay in 293 cells. (A) 293 cells were grown to different levels of confluency (100, 75 and 50%), transfected with a set of expression plasmids for pUL97 and incubated in either the presence (0–320 µM) or the absence of GCV. The GFP-expressing construct pCmn–GFP served as a negative control for which the transfection efficiency could be monitored under a fluorescence microscope. At 5 days post-transfection, plates could be evaluated visually for the degree of pUL97-mediated cytotoxicity. (B) For the optimization and quantification of signals, 293 cells (50% confluent) were transfected with the plasmids indicated and incubated with GCV (concentrations noted at the right). pUL97 kinase activity was quantified by measuring cytotoxicity signals (LDH activity) in the residual cell layers 5 days post-transfection. All measurements were based on triplicate transfections; in addition, LDH activity was determined in duplicate (six values in total). Mean and SD values are shown.

Completely negative for protein phosphorylation, M460I was still positive. Although phosphorylation was reduced to a lower efficiency (approximately tenfold), autophosphorylation of mutant M460I was clearly detectable. Moreover, further analysis provided initial evidence that protein phosphorylation of M460I was still sensitive to inhibition by indolocarbazole compounds (data not shown). These results illustrate that mutant K355M is negative for all kinase activities tested, while...
Inhibitors of HCMV pUL97 kinase activity

Fig. 3. Sensitivity of pUL97 to distinct protein kinase inhibitors. (A) For the UL97 in-cell-activity assay, 293 cells were transfected with the plasmids indicated and incubated with GCV (10–160 μM) in either the absence or the presence of the inhibitors NGIC-I, Go6976, Go7874 or AG-490 (50 nM). At 5 days post-transfection, LDH activity was determined in residual cell layers using the cytotoxicity assay. All measurements were performed in triplicate. Mean and SD values are shown. (B, C) For the UL97 in vitro kinase assay, 293 cells were transfected with the plasmids indicated and incubated in either the presence (250 nM) or the absence of the same inhibitors as above. pUL97 was immunoprecipitated from cell lysates and the kinase activity of precipitates was measured with respect to the phosphorylation of histone 2B and pUL97 (autophosphorylation). Separate values from one representative experimental series were determined by scanning the phosphorylation signals obtained on a phosphorimager blot. pcDNA-UL97 (a, b) was transfected and assayed in duplicate. The vector-transfected control (mock) is indicated.

mutant M460I retains some kinase activity and inhibitor sensitivity with respect to the wild-type protein.

Cell clones producing catalytically active pUL97 provide a tool for screening inhibitor compounds

We attempted to select 293 cell clones transfected with pcDNA-UL97, which should stably express high amounts of pUL97 kinase. Initially, however, we failed to maintain positive cell clones at higher passage numbers. Although several of the clones continued to express pUL97, as detectable by Western blot analysis, different tests for kinase activity were repeatedly negative. This seemed to suggest that the selection of inactive, spontaneously derived mutants of pUL97 was favoured under these conditions (data not shown). Thus, we developed a double-selection protocol for those clones expressing pUL97 kinase in an active state only. After transfection and selective growth of genetricin-resistant cells, individual clones were subjected to additional selection for the ability to convert GCV. Positive clones were cultivated and used for screening experiments. As an example, cell clone 293-UL97 F10 expressed kinase activity that was sensitive to the compound...
Fig. 4. Kinase activity of wild-type and mutant pUL97. (A) 293 cells were transfected with plasmids pcDNA-UL97 (wild-type; lane 2), pcDNA-UL97(K355M) (catalytically inactive mutant; lane 3), pcDNA-UL97(M460I) (GCV-resistant mutant; lane 4) or pcDNA3 (mock-transfected; lane 1). Cells were harvested 2 days post-transfection and analysed by Western blotting. As a control, HFF cells infected with HCMV strain AD169 for 3 days (lane 6) or mock-infected (lane 5) were assayed. Blots were developed using the pUL97-specific peptide antiserum PepAs 1343. The pUL97-specific band is marked on the left and molecular masses are indicated on the right. (B)–(D) 293 cells were transfected with the same plasmids as above, incubated with GCV (5–40 μM) in the presence of the solvent DMSO or the inhibitors NGIC-I or STP (5–500 nM). At 5 days post-
Inhibitors of HCMV pUL97 kinase activity

NGIC-I (Fig. 5 A). As a control, the vector-transfected cells (293-mock) did not produce kinase-specific signals (Fig. 5 B). The long-term passaging of different UL97-expressing cell clones eventually led to a decline in protein expression; for two independent cell clones, however, we could demonstrate that pUL97 remained clearly detectable for defined passage numbers and periods of analysis (Fig. 5 C). For periods of cultivation up to 2 months, pUL97 expression and activity were sufficiently high for kinase activity analysis and no change in the growth behaviour of the cultures was observed. Moreover, the inhibitor NGIC-I was regularly used as a control in individual screening experiments and showed identical properties of inhibition throughout the period of testing (data not shown). The generation of stably transfected cell lines presents a major improvement in the standardization and reliability for screening pUL97 activity, since the variables of individual transfections are eliminated. Moreover, the ease of handling of this cell system provides the opportunity to scale up each test panel. Thus, the results obtained with 293-UL97 cell clones confirm the data on pUL97-specific inhibition and deliver the basis for large-scale screening of pUL97 inhibitors.

Discussion

A novel strategy for interfering with HCMV replication during natural infection was postulated by the possibility of blocking the specific function of the viral protein kinase pUL97 (He et al., 1997). We describe a system for measuring viral protein kinase activity in cells in the presence of putative inhibitors. It was determined that indolocarbazole compounds exert a strong and specific inhibitory effect on pUL97 kinase activity, whereas cellular kinase functions are not notably impaired. Thus, pUL97 is an attractive target for novel antiviral drugs and the investigation of pUL97-specific kinase inhibitors offers insights into the regulatory role of pUL97 within the HCMV replication cycle.

For human herpesviruses, the nature of viral protein kinases was first recognized in the case of HSV-1 (DeWind et al., 1992) and the distinct functions of these viral protein kinases have been intensively investigated (Chee et al., 1989; Smith & Smith, 1989; Cunningham et al., 1992; Littler et al., 1992; Ng & Grose, 1992; Purves & Roizman, 1992; Sullivan et al., 1992; Ng et al., 1994, 1998; Daikoku et al., 1997; Ogler et al., 1997; Kawaguchi et al., 1998; Moffat et al., 1998; Ansari & Emery, 1999; Cannon et al., 1999; Chen et al., 2000). The role of HCMV-encoded protein kinase pUL97 in the virus replication cycle is not fully understood, although interaction with cellular proteins as phosphorylation targets was described previously (Kawaguchi et al., 1999). Furthermore, it has been demonstrated that pUL97 kinase activity is a critical factor for virus replication in tissue culture and that deletion of the UL97 gene results in severe replication deficiency (Prichard et al., 1999). In a recent study by Slater et al. (1999), the inhibitory effect of protein kinase inhibitors of the indolocarbazole class was demonstrated for HCMV infection in cultured human cells. However, the question of whether pUL97 kinase activity is directly impaired by these compounds was not addressed and so the antiviral mechanism remains speculative.

Indolocarbazoles were originally described as a chemical class of inhibitors of serine/threonine protein kinases of the PKC family which act by competitively blocking the ATP-binding site (reviewed by Goekjian & Jirousek, 1999). In order to analyse the possible inhibitory effect of indolocarbazole compounds on pUL97 in detail, novel experimental approaches were developed and, based on these findings, four main conclusions were drawn: (i) the activity of the viral protein kinase pUL97 is easily quantified in transfected cells in the absence of virus replication, (ii) the indolocarbazole compounds Go6976 and NGIC-I specifically block pUL97 kinase activity, (iii) the block in kinase activity is measurable by the level of GCV phosphorylation as well as protein phosphorylation, and (iv) GCV-resistant mutants of the UL97 gene (e.g. M460I), which encode a pUL97 kinase incapable of converting GCV, are not defective in expressing, at least in part, the kinase activity critical for protein phosphorylation. In conclusion, during replication of a UL97 mutant virus [e.g. virus variant AD169–GFP314 encoding UL97(M460I)], it seems that mutated pUL97 sufficiently fulfils its functional requirements. Considering the latter aspect, it is important to note that a GCV-resistant variant of HCMV [encoding mutant UL97(H520Q)] is also sensitive to inhibition by indolocarbazole compounds (Zimmermann et al., 2000). It will be interesting to see whether different virus variants and different cloned mutants of pUL97 possess different sensitivities towards indolocarbazoles, with respect to their GCV and protein phosphorylation activities (M. Marshall and others, unpublished data).

In general, the complete loss of UL97 function, achieved by genetic manipulation of the viral genome, causes a severe deficiency in HCMV replication in cell culture and virus titres of two to three orders of magnitude lower than those produced by the parental virus are attained (Prichard et al., 1999). In the present study, we provide evidence that specific inhibition of...
Fig. 5. 293-UL97 cell clones confirm the sensitivity of pUL97 to distinct protein kinase inhibitors. 293 cells were transfected with pCmn-UL97 or pcDNA3 (mock-transfected control) and subjected to double selection (geneticin plus GCV), as described in Methods. Clones 293-UL97 F10 (A) and 293-mock (B) were cultivated in the presence of GCV (10–320 µM) and NGIC-I (50 nM) or the solvent DMSO. At 5 days post-incubation, the colour change in the culture media was quantified by direct photometric determination. Mean and SD values are shown. (C) In parallel, clones 293-UL97 F10 and 293-mock (upper panels) as well as 293-UL97Axx and 293-mockAxx (lower panels; transfected with plasmid pLXSN-UL97 or vector pLXSN, respectively) were assayed on Western blots for the expression of pUL97. For clone 293-UL97 F10, samples were taken at passage numbers 4 (lane 2) and 16 post-transfection (lane 4; compare with lane 1 containing untransfected 293 cells and lane 3 containing clone 293-mock). For clone 293-UL97Axx, samples were taken either immediately (lane 6) or 22 days post-transfection (lane 8; compare with lanes 5 and 7 containing clone 293-mockAxx immediately post-transfection or 22 days post-transfection). Blots were developed using UL97-specific antibodies (lanes 1–4, MAb-UL97; lanes 5–8, PepAs 1343).

pUL97 kinase activity can be achieved by treatment with indolocarbazole compounds. So far, it is unknown whether inhibition of pUL97 kinase activity by indolocarbazoles is solely and directly responsible for the block in virus replication. Nevertheless, our study demonstrates that some indolocarbazole compounds possess strong activity against pUL97 in the absence of major cellular side-effects. The avoidance of interfering with cellular kinase functions might be critical for the success of antiviral treatments directed towards viral protein kinases. However, it should be mentioned that inhibition of cellular protein kinases, as investigated in the case of human immunodeficiency virus type 1 (HIV-1), can result in a strong antiviral effect. It was reported previously that the indolocarbazole compound Go6976 acts as a potent antagonist of virus reactivation in latently infected cells (Quatsha et al., 1993) and that interference with regulatory cellular pathways...
might be responsible for this block. Therefore, it was speculated that inhibition of the kinase-mediated activation of the NF-κB transcription factor was a critical step in the anti-HIV-1 mechanism and the importance of this aspect was underscored in a further study on NF-κB antagonists (Mhashilkar et al., 1997). By analogy, the activity of NF-κB plays a critical role in the cellular regulation of herpesvirus replication, as described in detail for HCMV (Kowelik et al., 1993; Yurochko et al., 1995, 1997) and HSV-1 (Hanson et al., 1998). In contrast to the situation in HIV-1 reactivation, HSV-1 replication in cell culture shows no significant sensitivity to G66976 (Slater et al., 1999; M. Marschall and others, unpublished results). Thus, the antiviral effect of G66976 on members of the herpesvirus family such as HCMV is selective and is based on virus-specific rather than cell-specific mechanisms. Consequently, we postulate that inhibition of HCMV protein kinase pUL97 is the predominant mechanism responsible for the strong antiviral effect of the indolocarbazole compounds described here. During the preparation of this manuscript, a study was published that underlines the inhibitory capacity of indolocarbazoles at the level of HCMV replication as well as pUL97 kinase activity, and the data are in agreement with our findings (Zimmermann et al., 2000). Future studies, possibly performed on indolocarbazole-resistant HCMV mutants, will have to demonstrate the causative linkage of these two effects and to illuminate the value of these findings for antiviral research and novel strategies in antiviral therapy.

This work was supported by the BMBF (grant no. 0311738A) and IZKF Erlangen. The authors are grateful to Professor Dr T. Mertens and collaborators (University of Ulm, Germany) for the gift of UL97-specific antibodies, to Martina Kronschnabl and Peter Lischka for many helpful discussions and to Professor Dr B. Fleckenstein for continuous support.

References


Received 23 November 2000; Accepted 2 February 2001