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Direct targeting of human cytomegalovirus protein kinase pUL97 by kinase inhibitors is a novel principle for antiviral therapy

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The protein kinase pUL97, encoded by human cytomegalovirus (HCMV), is an important determinant of virus replication. Recently, indolocarbazoles were identified as a class of substances that inhibit the pUL97 kinase activity *in vitro*. In parallel, it was shown that indolocarbazoles interfere with HCMV replication; however, the causal relationship between inhibition of pUL97 kinase activity and virus replication has not been clarified. Here evidence is provided that indolocarbazole-mediated inhibition of virus replication is a direct result of diminished pUL97 protein kinase activity. In cell culture infections, a strong and selective antiviral activity was measured with respect to several strains of HCMV in contrast with other related or non-related viruses. For fine quantification, recombinant HCMVs expressing green fluorescent protein were used, demonstrating the high sensitivity towards compounds NGIC-I and Gö6976. Interestingly, a ganciclovir-resistant virus mutant (UL97-M460I) showed increased sensitivity to both compounds. Supporting this concept, transfection experiments with cloned pUL97 revealed that ganciclovir-resistant mutants were characterized by reduced levels of autophosphorylation compared with wild-type and possessed particularly high sensitivity to indolocarbazoles. Moreover, the Epstein–Barr virus-encoded homologous kinase, BGLF4, which showed a similar pattern of autophosphorylation and ganciclovir phosphorylation activities, was not inhibited. Importantly, a cytomegalovirus deletion mutant, lacking a functional UL97 gene and showing a severe impairment of replication, was completely insensitive to indolocarbazoles. Thus, our findings indicate that a specific block in the activity of pUL97 is the critical step in indolocarbazole-mediated inhibition of virus replication and that pUL97 might be targeted very efficiently by a novel antiviral therapy.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen causing a variety of diseases in man. Although the infection of immunocompetent adults is mild or asymptomatic in most cases, severe clinical problems frequently arise from the infection of the fetus or immunocompromised individuals. In such cases, HCMV disease is manifested in a generalized form or in several organs and tissues, such as the lung, gastrointestinal tract, retina, CNS, liver or heart (reviewed by Pass, 2001).

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Currently, antiviral therapy is mainly based on inhibitors of viral DNA synthesis, such as ganciclovir (GCV), foscarnet (FOS), cidofovir (CDV) and the prodrugs valganciclovir (reviewed by Curran & Noble, 2001) and valacyclovir (Lowance *et al.*, 1999). In contrast to FOS, which directly interferes with the viral DNA polymerase, nucleoside analogues such as GCV have to be converted by the viral kinase pUL97 into monophosphorylated metabolites, which are further phosphorylated by cellular kinases into the active form. Clinical treatment with these antivirals, however, is accompanied by adverse side effects, such as myelo- or nephrotoxicity. In addition, the success of therapy may be threatened by the rapid selection of drug-resistant virus variants (reviewed by Crumpacker, 2001; Emery & Griffiths, 2000; Limaye *et al.*, 2000). Novel strategies of antiviral therapy are directed to

other essential steps in virus replication (Efferth *et al.*, 2002; reviewed by Scholz *et al.*, 2001), for example to the onset of transcription of the immediate early region (antisense oligonucleotide ISIS 2922; reviewed by Perry & Balfour, 1999) or the processing of concatemeric genome precursors (terminase inhibitors of the benzimidazole class such as BDCRB and TCRB; reviewed by Chulay *et al.*, 1999). Another benzimidazole derivative, 1263W94, has recently been characterized by its strong potential to inhibit GCV-sensitive and -resistant HCMVs (McSharry *et al.*, 2001). In this regard, initial findings suggest that 1263W94 may target the pUL97 function, but the limited amount of data await further investigation (Davis *et al.*, 1998).

We have studied the viral protein kinase pUL97 for its potential use in antiviral strategies, since we and others have recently reported the identification of highly efficient inhibitors of the pUL97 kinase activity (Marschall *et al.*, 2001a; Zimmermann *et al.*, 2000). These inhibitors, belonging to the chemical class of indolocarbazoles, were first characterized by their inhibitory activities directed towards members of the protein kinase C (PKC) family of cellular protein kinases (reviewed by Goekjian & Jirousek, 1999). Moreover, they were described as potent inhibitors of HCMV replication (Slater *et al.*, 1999; Zimmermann *et al.*, 2000). However, the direct link between inhibition of a viral protein kinase and interference with virus replication has not so far been clarified.

Several studies have shown that the viral protein kinase pUL97 is particularly important for efficient replication (Michel *et al.*, 1996; Prichard *et al.*, 1999; Wolf *et al.*, 2001). Deletion of the UL97 gene in the viral genome was found to be critical for the infection of cultured cells, resulting in a drastic decline in virus replication. Searching for the natural function of pUL97 within the virus replication cycle, it was speculated that pUL97 may be involved in the activation of phosphorylation-dependent viral regulatory proteins, but only a limited amount of data has been published concerning the identification of phosphorylation targets. A second activity of pUL97, however, is well characterized, i.e. the phosphorylation of therapeutically important nucleoside analogues, such as GCV (Sullivan *et al.*, 1992; Littler *et al.*, 1992) and acyclovir (Talarico *et al.*, 1999). Importantly, it was shown that pUL97 is not a natural nucleoside kinase (Michel *et al.*, 1996) and might therefore not directly, but possibly indirectly, be involved in nucleotide metabolism or DNA synthesis.

The presented study provides evidence that the HCMV-encoded protein kinase pUL97 is the target responsible for the strong antiviral effect of indolocarbazoles. This was clearly demonstrated for wild-type as well as laboratory strains, mutant and recombinant variants of HCMV, while a UL97 deletion mutant and other viruses tested were insensitive. It has to be stressed that GCV-resistant HCMV showed particularly high indolocarbazole sensitivity. This might open new possibilities for the clinical treatment of infections with GCV-resistant and -sensitive HCMV.

Methods

■ **Cell culture and viruses.** Primary human foreskin fibroblasts (HFFs), 293, Vero and MDCK cells were cultivated in minimal essential medium (MEM or DMEM) containing 5% foetal calf serum and 100 μg/ml gentamicin. HCMVs AD169 (laboratory strain) and TB40E (endotheliotropic strain), the recombinant viral clone containing the green fluorescent protein (GFP) AD169-GFP and the GCV-resistant mutant AD169-GFP314 (Marschall *et al.*, 2000) were propagated in HFFs. The UL97-deleted virus clone AD169delUL97 was subjected to selection with 200 μM mycophenolic acid and 5 μM xanthine before plaque purification prior to use in infection experiments (Prichard *et al.*, 1999). Virus titres were determined by plaque formation (p.f.u.) or GFP expression (GFP-TCID), respectively. Herpes simplex virus type 1 (HSV-1) was isolated from clinical material and propagated in Vero cells. Human influenza A/WSN/33 virus was grown in embryonated hen eggs or MDCK cells and titrated by haemagglutination. Aliquots of all viruses were stored at −80 °C.

■ **Protein kinase inhibitors and antiviral compounds.** Indolocarbazole compounds staurosporine (STP), Gö6976, Gö7874 and NGIC-I (reviewed by Goekjian & Jirousek, 1999) were purchased from Calbiochem. Stock solutions were prepared in DMSO and stored at −20 °C. Ganciclovir (GCV, Cytovene, pharmaceutical grade, Syntex Arzneimittel/Roche) and cidofovir (CDV, Vistide, Pharmacia & Upjohn) were used as stocks in aqueous solution.

■ **Cloning of UL97 and BGLF4.** Mutants of ORF-UL97 encoding pUL97(M460I), pUL97(L595S), pUL97(C603W) and pUL97(H469V) were derived from GCV-resistant viruses. In previous reports, the first three of these UL97 mutations have been described as resistance-conferring determinants, as confirmed by site-directed mutagenesis or marker transfer experiments (reviewed by Erice, 1999). Another published mutation (H469Y), which is similar to the latter mutant used here, was only recently described as resistance-associated, but this has not yet been confirmed by a marker transfer (Jabs *et al.*, 2001). The mutant pUL97(K355M) is catalytically inactive, due to an amino acid exchange at the invariant lysine 355 of the ATP binding site, and has been described before (Marschall *et al.*, 2001a). In addition, the mutant pUL97(S180A) was chosen, since S180 has been identified as a candidate for the main target site of pUL97 autophosphorylation (M. Stein-Gerlach, unpublished results). All constructs were generated with vector pcDNA3.1 (Invitrogen). Expression constructs pcDNA-UL97, pcDNA-UL97–FLAG, pcDNA-UL97(K355M) and pcDNA(M460I) have been described elsewhere (Marschall *et al.*, 2001a). pcDNA-UL97(C603W), pcDNA-UL97(H469V) and pcDNA-UL97(L595S) were constructed after PCR amplification of the ORF-UL97 from GCV-resistant clinical isolates of HCMV. PCR was performed using Vent DNA polymerase (New England BioLabs) in 35 cycles, each composed of 40 s at 95 °C for denaturation, 40 s at 50 °C for annealing and 120 s at 72 °C for polymerization. BGLF4 was amplified by PCR using Epstein–Barr virus (EBV) genomic DNA of reference strain B95-8 as a template and cloned in fusion to the C-terminal FLAG sequence (GACTACAAAGACGATG-ACGACAAG) of the modified vector pcDNA3.1–FLAG. pcDNA-UL97(S180A) was generated using the wild-type construct pcDNA-UL97 to perform site-directed mutagenesis (Kunkel, 1985) applying a mutagenesis primer (Mut180-UL97, TCCGAGGGGTCGGCGCCGCC-GGTGAA) in order to substitute the codon 180 AGC (serine) by GCC (alanine). PCR primers used were: 5′ UL97-*Eco*RI CCCGAATTCATGT-CCTCCGCACTTCGG, 3′ UL97-*Xho*I CCGCTCGAGTTACTCGGGG-AACAGTTG, 5′ BGLF4-*Eco*RI TGAGAATTCATGGATGTGAATAT-GGCTGCGGAG, 3′ BGLF4-*Xba*I TGATCTAGATCCACGTCGGCC-ATCTGGACC.

Table 1. Indolocarbazoles: pUL97 kinase inhibition and levels of cytotoxicity

Indolocarbazole compound	Mean inhibitory concn IC_{50}–pUL97 [nM]*	Mean cytotoxic concn CTX_{50}–HFF [nM]†
STP	651·7 ± 37·5	9·8 ± 0·5
Gö6976	240·4 ± 32·5	> 100
NGIC-I	42·8 ± 8·0	> 100
Gö7874	> 1000	> 100

* For UL97 *in vitro* kinase assays, pUL97 was immunoprecipitated from transfected 293 cells and analysed for histone 2B phosphorylation in the presence of indolocarbazoles. Data were produced in duplicate for serial concentrations from 10 nM to 1000 nM and mean values (± SD) are given.
† For determination of cytotoxicity, HFF cells were incubated with indolocarbazoles at concentrations of 1, 10, 50 and 100 nM and cultivated for 7 days. Mean LDH activity (samples in quadruplicate) was measured with lysates prepared from the residual cell layers.

■ **UL97 in-cell-activity assay.** A novel quantification system for the GCV phosphorylation activity of pUL97 has been established (Marschall *et al.*, 2001a). The assay is based on the fact that GCV conversion by pUL97 leads to the induction of strong cytotoxic effects, so that pUL97 kinase activity can be easily quantified in UL97-transfected cells as a function of cytotoxicity. In brief, 293 cells were used for lipofectamine transfection with pUL97 expression constructs in a 96-well format. GCV was incubated in a gradient of serial concentrations and protein kinase inhibitors were added immediately. After cultivation for 5 days, photometric quantification became possible due to the colour change (yellow to red) in the phenol red-containing culture medium and was performed by the use of an ELISA plate reader (A_{560}).

■ **UL97 *in vitro* kinase assay.** The kinase activity of pUL97 was determined *in vitro* after recombinant expression in transfected 293 cells and immunoprecipitation of the kinase from cell lysates, as described recently (Marschall *et al.*, 2001a). Putative inhibitors, such as the indolocarbazole compounds, were either added to the culture medium 2 h before lysis (see Fig. 3b, d) or added directly to the kinase reaction (see Table 1).

■ **Cytotoxicity assay.** HFF cells were cultivated in 12-well plates and test compounds were incubated in the medium at 37 °C for 7 days. Lactate dehydrogenase (LDH) activity in the residual cell layer was measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) as described elsewhere (Efferth *et al.*, 2002).

■ **Plaque reduction assay (PRA).** Standard conditions for the PRA have been described before (Marschall *et al.*, 2000). Agarose overlays, 0·3% (w/v), were supplemented with a dilution of one of the respective test compounds. The plates were incubated at 37 °C for 8–12 days before plaque formation was visualized by staining with 1% crystal violet.

■ **HCMV GFP-based antiviral assay.** The assay was established by the use of recombinant HCMVs expressing GFP (Marschall *et al.*, 2000). For this, HFF cells were cultivated in 12-well plates to 90% confluency and used for infection with AD169-GFP or AD169-GFP314 virus at a GFP-TCID of 0·25 (equivalent to 25% GFP-positive cells at 7 days post-infection). Infected cell layers were incubated, optionally with one of the respective inhibitor compounds, at 37 °C in a 5% CO_2 atmosphere for 7 days. Lysates of the infected cells were prepared and used for automated measurement of GFP signals in a Victor 1420 Multilabel Counter (Perkin Elmer Wallac).

■ **Flow cytometric analysis (FACS).** HFF cells were grown on 6-well plates, infected with GFP-expressing HCMV and cultivated in the presence or absence of inhibitory substances. Then cells were harvested, fixed in solution with 3% formaldehyde and directly analysed for GFP fluorescence by flow cytometry as described before (Marschall *et al.*, 2000).

■ **Western blot.** Protein samples were denatured under reducing conditions and SDS–PAGE was performed according to standard protocols. Blots were incubated with a pUL97-specific rabbit antiserum [PepAs 1343; raised with immunization peptide UL97/1-16 (MSSALR-SRARSASLGT)] or MAb-FLAG (M2; Sigma) and peroxidase-conjugated secondary antibodies (Dianova). Signals were developed in an enhanced chemiluminescence reaction (ECL Western Detection kit; Amersham Pharmacia) according to the manufacturer's instructions.

Results

Protein kinase inhibitors show high activity against viral pUL97 but do not induce cytotoxicity in human fibroblasts

The protein phosphorylation activity of cloned pUL97 was analysed by the UL97 *in vitro* kinase assay. Here, both autophosphorylation and the phosphorylation of exogenously added histone 2B (known as an *in vitro* substrate of pUL97) were measured in the absence or presence of inhibitors. STP, as a lead compound, and the derivatives NGIC-I, Gö6976 and Gö7874, all possessing strong inhibitory activity against members of the PKC family, were investigated for pUL97 specificity (Fig. 1a). These four compounds were chosen after a large-scale screening for UL97-specific kinase inhibitors, since they showed strongly varied inhibitory potentials. A detailed analysis demonstrated that NGIC-I was the most effective compound in the inhibition of histone 2B phosphorylation, being characterized by an IC_{50} of 42·8 ± 8·0 nM (Table 1). Gö6976 also showed strong inhibitory capacity, while STP was only moderately active and Gö7874 was negative. Inhibition was measured in the direct presence of compounds in the *in vitro* kinase assay and the inhibitory effect of Gö6976 and NGIC-I in nanomolar concentrations was outstanding with respect to all other compounds tested. Results were mostly identical in experiments measuring both phosphorylation of histone 2B and autophosphorylation (data not shown). Thus, the *in vitro* kinase assays identified indolocarbazoles, primarily NGIC-I, as potent inhibitors of pUL97 activity.

Furthermore, the effects of these indolocarbazoles on the viability of cultured cells were determined (Table 1). HCMV-permissive HFFs were subjected to a treatment with indolocarbazoles for 7 days. The range of concentrations (1–100 nM) was chosen to cover those concentrations applied in infection

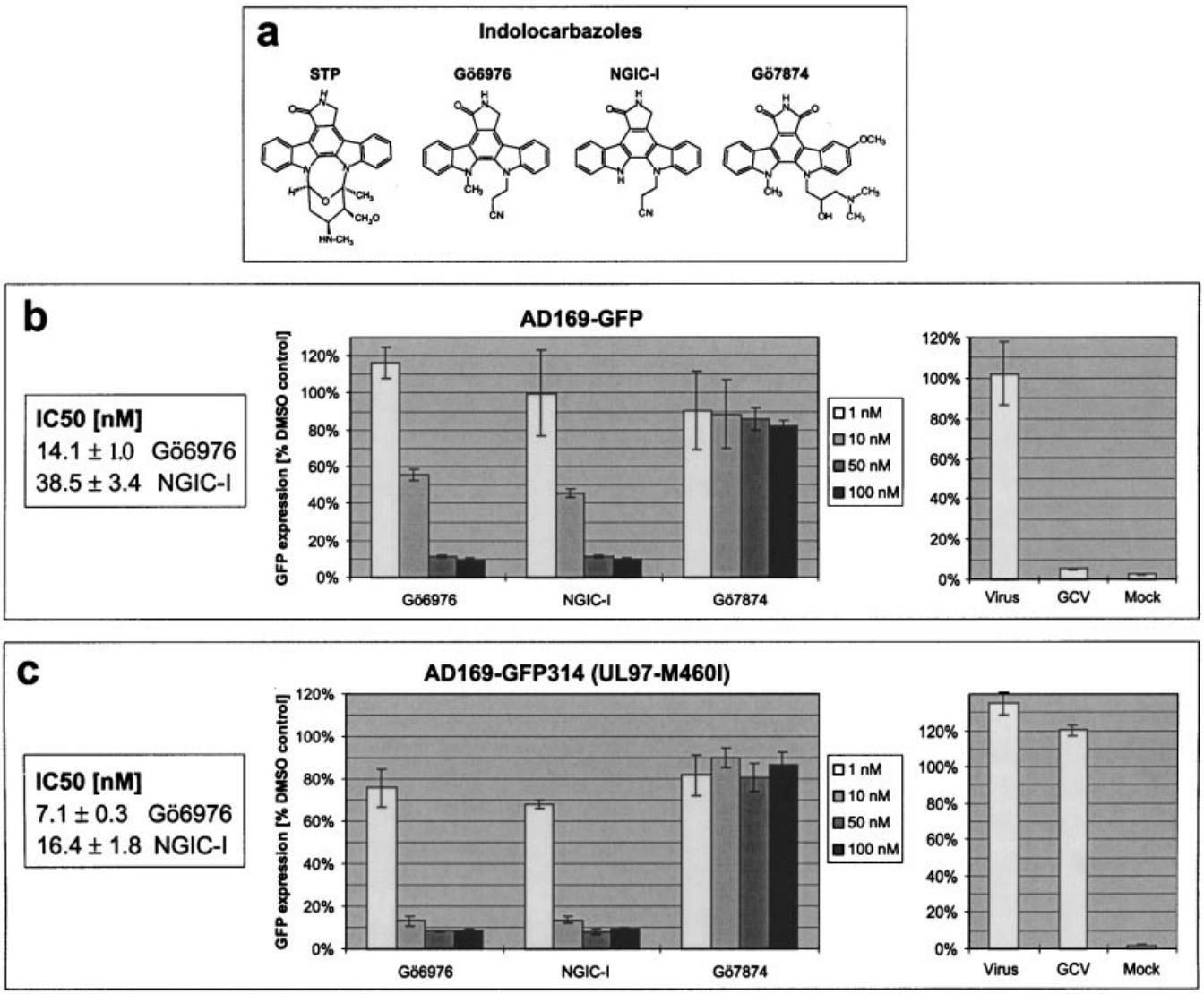

Fig. 1. Inhibition of HCMV replication by indolocarbazoles. Chemical structures of indolocarbazoles: the natural product STP and three non-glycosidic derivatives (a). For quantification of HCMV replication (GFP-based antiviral assay), HFF cells were cultivated in 12-well plates, infected with GCV-sensitive AD169-GFP virus (b) or GCV-resistant AD169-GFP314 virus (c) and treated with various concentrations of indolocarbazoles (1, 10, 50 and 100 nM, left panels) or GCV (10 μM, right panels). Seven days post-infection, cells were assayed for GFP expression by automated fluorimetry. GFP expression was taken as 100% for infection without inhibitor (DMSO control) and relative mean values of triplicate determinations are shown. The concentrations resulting in 50% inhibition (IC_{50}) are presented on the left.

experiments (see Figs 1, 2 and 4). In the cytotoxicity assay, no negative effect on cell viability was detectable for the compounds NGIC-I, Gö6976 and Gö7874, while STP induced high cytotoxicity. Thus, NGIC-I and Gö6976 were considered as interesting virus-specific candidates for inhibitors, lacking the induction of unwanted cellular side-effects.

Indolocarbazoles are potent inhibitors of HCMV replication, both for GCV-sensitive and GCV-resistant virus strains

Antiviral activity of indolocarbazole compounds was determined by the fine-quantification method using GFP-expressing recombinant HCMVs (GFP-based antiviral assay). For AD169-GFP virus, a strong inhibitory effect of NGIC-I and Gö6976 was measured, while the compound Gö7874 did not inhibit virus replication. The IC_{50} values for NGIC-I and Gö6976 were $38{\cdot}5 \pm 3{\cdot}4$ nM and $14{\cdot}1 \pm 1{\cdot}0$ nM, respectively (Fig. 1b; IC_{50} calculation was based on additional experiments using serial concentrations between 10 nM and 50 nM). Importantly, only those compounds effectively inhibiting the *in vitro* kinase activity of pUL97 were highly active in the inhibition of HCMV replication (NGIC-I and Gö6976), while other compounds, such as Gö7874 and a number of other known protein kinase inhibitors (data not shown) had no effect.

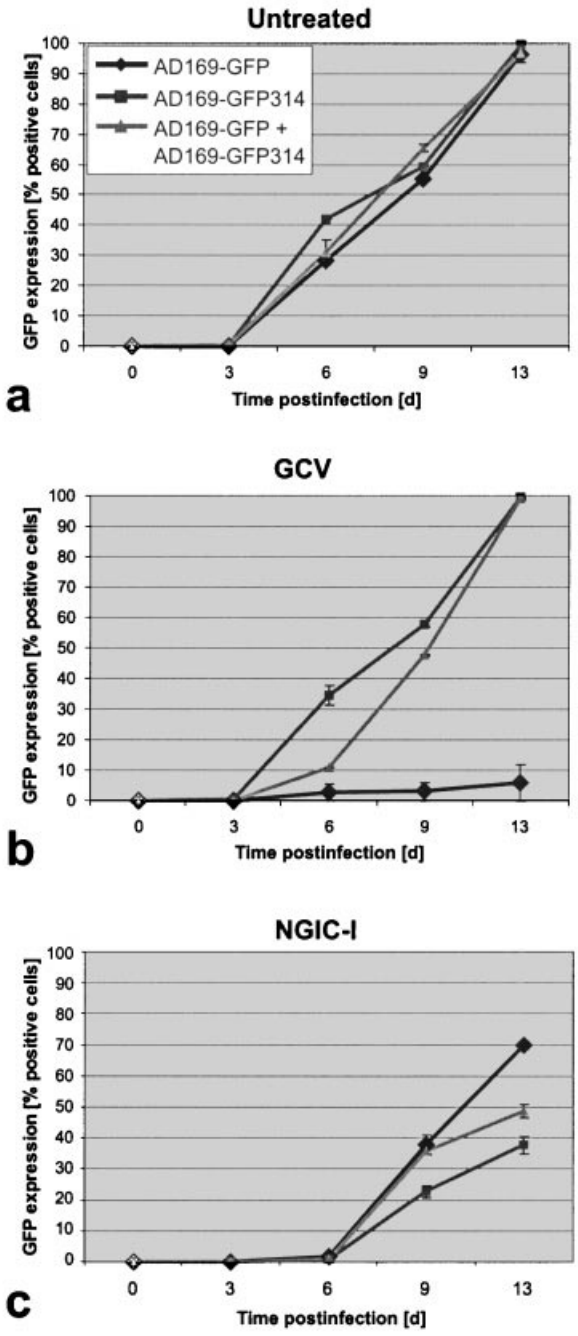

Fig. 2. Mutant virus fitness and drug sensitivity. A flow cytometric analysis (FACS) was performed to study the kinetics of HCMV replication under treatment with antivirals. HFF cells were grown on 6-well plates, infected with AD169-GFP virus, the GCV-resistant virus mutant AD169-GFP314 or a mixed inoculum of both viruses (duplicate samples). Subsequently, infected cells were cultivated in the presence or absence of inhibitory substances: (a) untreated; (b) 10 μM of GCV; (c) 50 nM of NGIC-I. At various times post-infection (3, 6, 9 and 13 days), cells were harvested and analysed for GFP fluorescence. The percentages of GFP-positive cells per total cell number were determined by two independent measurements and mean values are given (±SD, for some values below detection limit). A second replicate of experiments produced identical results (not shown).

In parallel, a GCV-resistant virus mutant, AD169-GFP314 (UL97-M460I), was tested under identical conditions (Fig. 1c). GCV resistance of AD169-GFP314 is visualized in the panel on the right of Fig. 1(c). Its resistance factor was calculated as 4·7 ± 0·8 (Schmidt *et al.*, 2000; fold resistance based on GCV IC_{50} compared with wild-type virus, data not shown). Interestingly, this virus mutant possessed increased sensitivity to NGIC-I as well as Gö6976 (IC_{50} values 16·4 ± 1·8 nM and 7·1 ± 0·3 nM, respectively). Clearly, at 10 nM concentrations, the levels of virus replication were distinct from those of the parental virus AD169-GFP, and when tested repeatedly, we detected significant differences in drug sensitivity between the two viruses within the range of concentrations from 5 nM to 20 nM. In addition, an independently isolated GCV-resistant variant of HCMV, AD169-GFP26 (also carrying mutation UL97-M460I) was tested and produced very similar results (IC_{50} 14·0 ± 2·4 for NGIC-I and 6·3 ± 0·7 for Gö6976; data not shown). This indicates that an antiviral strategy targeting the pUL97 protein kinase activity might be promising for wild-type and mutant HCMV.

In a second experimental approach, the drug sensitivity of these viruses was confirmed by a replication kinetic analysis using GFP-based flow cytometry. In infected cells without the addition of drugs, the virus mutant AD169-GFP314 replicated as efficiently as AD169-GFP (Fig. 2a). This demonstrated that the virus mutant did not possess a reduced fitness of *in vitro* replication. The finding was surprising, considering the fact that an approximately 10-fold reduced level of pUL97 autophosphorylation was identified for mutation UL97-M460I (Marschall *et al.*, 2001a; see also Fig. 3b). Under GCV treatment, the GCV-resistant phenotype of mutant AD169-GFP314 became apparent by the unchanged, high level of replication, which stood in clear contrast with the strong inhibition of AD169-GFP (Fig. 2b). Upon coinfection with both viruses, the wild-type behaviour (inhibition) was seen in the first phase, while the phenotype of the mutant (resistance) became predominant in the second phase after 9 days. In the presence of NGIC-I, the replication of both viruses was completely inhibited up to day 6, after which the activity of the indolocarbazole compound appeared to decline. Subsequently, virus inhibition ceased in a manner such that the parental virus was no longer inhibited while the mutant AD169-GFP314 still showed a clearly measurable level of sensitivity to NGIC-I (Fig. 2c). The coinfection with both viruses resulted in an intermediate phenotype. This confirmed the increased indolocarbazole sensitivity of mutant AD169-GFP314 and, combined, these findings suggested that point mutations in UL97 might render GCV-resistant HCMV particularly sensitive to pUL97-targeting drugs.

Wild-type and mutant proteins of UL97 vary in their kinase activities and in their sensitivities to inhibitors

The central question addressing the specificity and efficacy of indolocarbazoles was further investigated by transfection

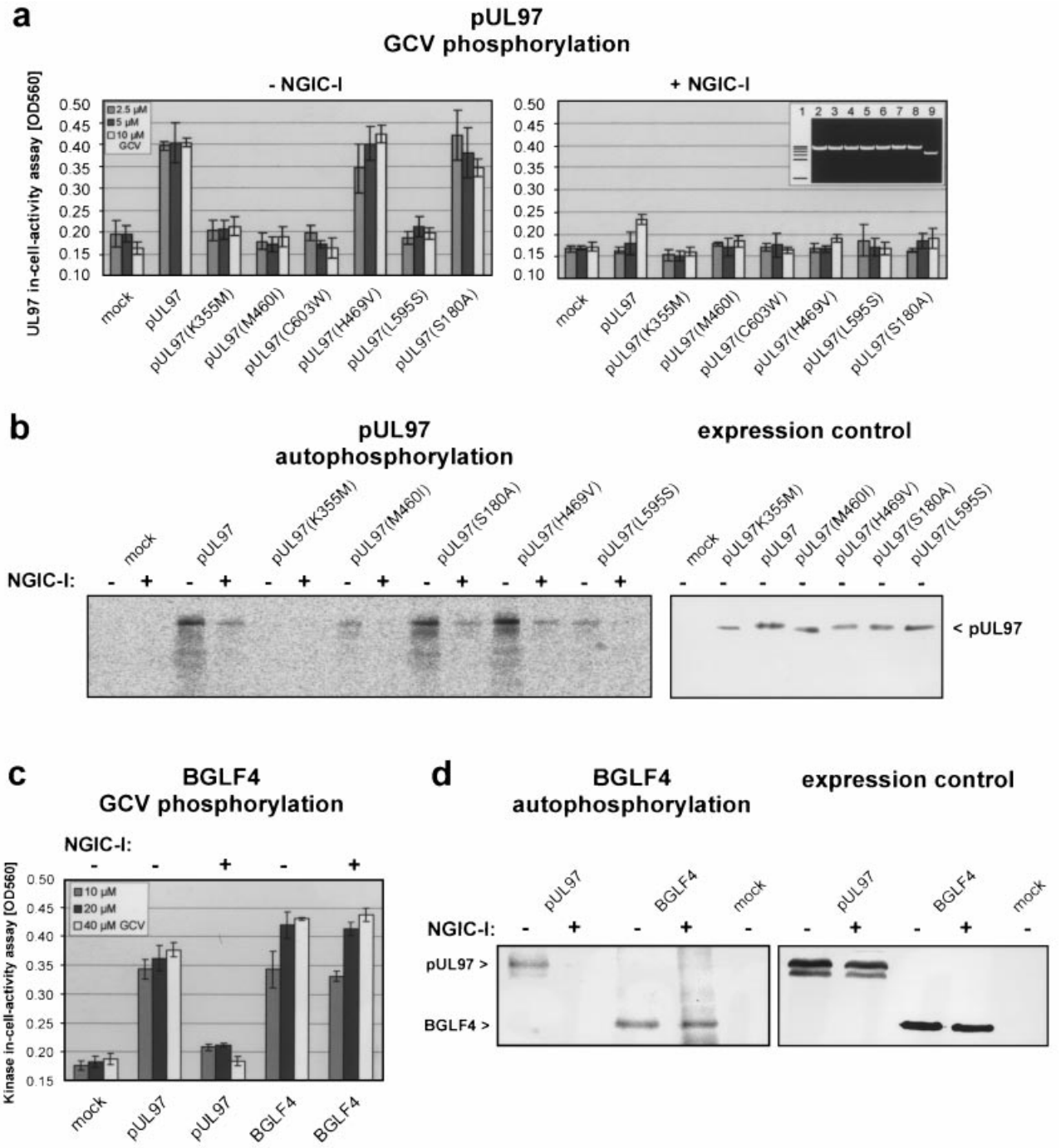

Fig. 3. GCV phosphorylation and autophosphorylation: comparison of wild-type pUL97, mutants and a related viral kinase. (a) A series of plasmid constructs was used for the expression of wild-type and mutants of pUL97. Plasmids were linearized by restriction digest and exactly equilibrated to identical concentrations for use in transfections, as shown on agarose gel as insert in the right panel: (1) marker; (2) pcDNA-UL97; (3) pcDNA-UL97(K355M); (4) pcDNA-UL97(M460I); (5) pcDNA-UL97(C603W); (6) pcDNA-UL97(H469V); (7) pcDNA-UL97(L595S); (8) pcDNA-UL97(S180A); (9) pcDNA3.1. GCV phosphorylation was measured in transfected 293 cells (UL97 in-cell-activity assay; 2·5, 5 and 10 µM of GCV). Cultivation of the transfected cells was performed in the presence (+) or absence (−) of 100 nM of NGIC-I. Measurements were performed in triplicate and mean values are shown. (b) Autophosphorylation was measured with pUL97 immunoprecipitated from transfected 293 cells (UL97 *in vitro* kinase assay; left panel). Cultivation of the transfected cells was performed in the presence (+) or absence (−) of 500 nM of NGIC-I. Data of the phosphoimaging blot of one representative experiment are shown. The levels of expressed proteins were detected by Western blotting as demonstrated in the right panel (pUL97-specific antiserum PepAs 1343). (c) GCV phosphorylation of the EBV protein kinase BGLF4 was compared with pUL97 (10, 20 and 40 µM of

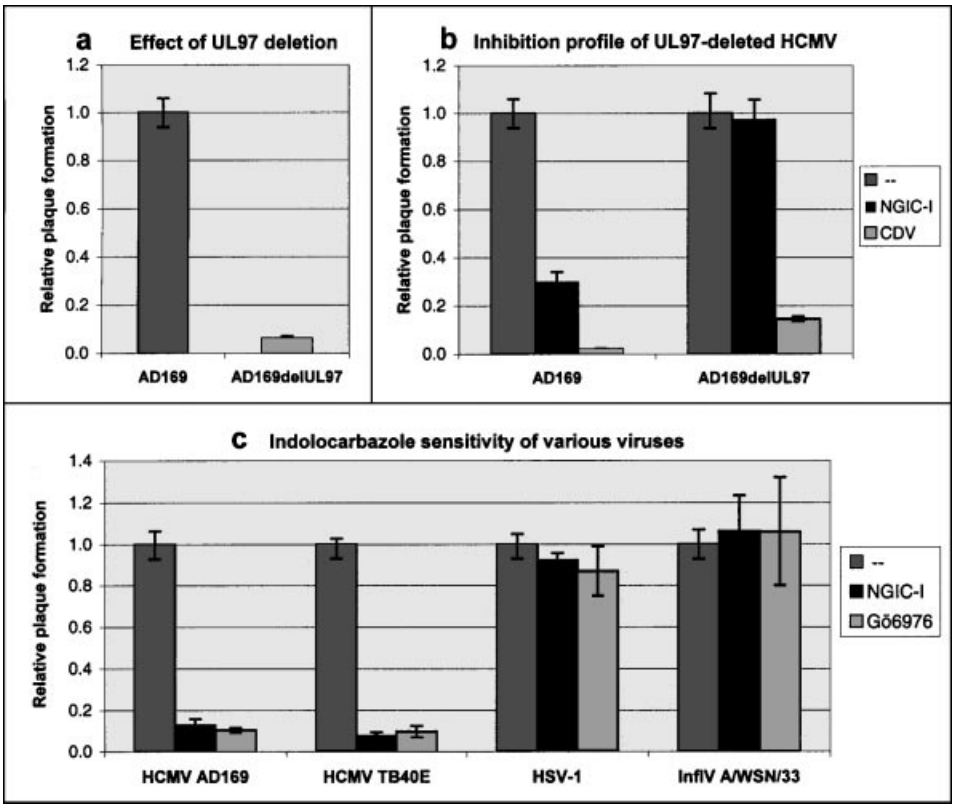

Fig. 4. Replication and inhibitor sensitivity of HCMV encoding wild-type UL97, HCMV with a UL97 deletion and other viruses. (a) HCMV AD169 and the UL97-deleted mutant AD169delUL97 were used for infection of HFF cells in 12-well plates. Progeny virus production was quantified by plaque formation. Note the drastically reduced replication efficiency of AD169delUL97 virus. (b) For determining the characteristics of drug sensitivity, relative plaque formation of both viruses was measured in the presence or absence of NGIC-I (50 nM) or CDV (1 μM). (c) For comparison with related and non-related viruses, permissive cells were cultivated in 12-well plates (HFF, Vero and MDCK, respectively) and used for infection with HCMV AD169, HCMV TB40E, HSV-1 (clinical isolate) and human influenza A/WSN/33 virus, respectively. Infected cells were treated with 50 nM of indolocarbazoles (NGIC-I or Gö6976) or solvent DMSO as a control (–). Plaque numbers for all panels of this figure were counted twice from infections performed in duplicate and mean values (±SD) are presented.

experiments with pUL97 wild-type and point mutants. In a first series of experiments, we determined the GCV phosphorylation activity of these mutants. GCV phosphorylation was studied by our recently established UL97 in-cell-activity assay. For this, transfection of 293 cells was performed with exactly calibrated concentrations of UL97-expressing plasmids (Fig. 3a, see insert of right panel). Strong GCV phosphorylation activity was detected for wild-type pUL97, as well as mutants pUL97(H469V) and pUL97(S180A). In this sensitive test system, the two mutants were, surprisingly, indistinguishable from wild-type and were associated with neither GCV-resistance nor with an altered level of activity. In contrast, mutants pUL97(M460I), pUL97(L595S) and pUL97(C603W) did not show GCV phosphorylation in the applied range of concentrations, reflecting the expected GCV-resistant phenotype. Mutant pUL97(K355M) was used as a catalytically inactive control. As far as the indolocarbazole sensitivity was concerned, a significant reduction of signals for wild-type pUL97 as well as mutants pUL97(H469V) and pUL97(S180A) was measured upon treatment with NCIC-I (100 nM). This indicates an inhibition of pUL97 kinase activity that is independent of the influence of these point mutations.

Autophosphorylation was analysed by the UL97 *in vitro* kinase assay (Fig. 3b, left panel) and expression levels were monitored by Western blot analysis (Fig. 3b, right panel). Autophosphorylation was detectable for wild-type pUL97 and all mutants, except for negative control pUL97(K355M). These results underline previous findings indicating that autophosphorylation activity of pUL97 mutants is distinct from GCV phosphorylation and that all GCV-resistant mutants

GCV) in transfected 293 cells (plasmids pcDNA-BGLF4–FLAG, pcDNA-UL97–FLAG and pcDNA3.1). (d) Autophosphorylation of BGLF4 was determined with FLAG-tagged protein immunoprecipitated from transfected 293 cells (MAb-FLAG; left panel). Cultivation of the transfected cells was performed in the presence (+) or absence (−) of 500 nM of NGIC-I. The levels of expressed proteins were detected by Western blot analysis using MAb-FLAG (right panel).

analysed so far retained, at least in part, autophosphorylation activity. Importantly, however, the GCV-resistant mutants pUL97(M460I) and pUL97(L595S) showed a significantly lower level of autophosphorylation compared to wild-type. In contrast, the two GCV-sensitive point mutants, pUL97(H469V) and pUL97(S180A), were very similar to wild-type. Indolocarbazole sensitivity was analysed by the treatment of pUL97-transfected cells with NGIC-I (500 nM). All mutants and pUL97 wild-type were significantly inhibited by NGIC-I. It was striking that in the case of the two GCV-resistant mutants, inhibition of autophosphorylation was most pronounced, with a drop in autophosphorylation signals close to the detection limit (Fig. 3b). The third GCV-resistant mutant, pUL97(C603W), was likewise highly NGIC-I-sensitive but could not be included in the quantification due to an unexpectedly high level of protein synthesis (even after repeated, exact adjustment of the concentration of transfected DNA in reproduction experiments; data not shown). It seemed to suggest that mutation C603W alters protein stabilty and/or expression efficiency in transfected cells. Wild-type pUL97 and the two GCV-sensitive point mutants, pUL97(H469V) and pUL97(S180A), on the other hand, showed a lesser degree of inhibition compared with GCV-resistant mutants. Thus, this finding proposes a mechanism for the increased indolocarbazole effect of GCV-resistant virus: GCV-resistant mutants of pUL97, showing a reduced level of autophosphorylation per se, might generally possess higher sensitivities to pUL97-specific inhibitors such as NGIC-I. Furthermore, no cross-resistance between GCV and indolocarbazoles was identified by our analysis.

To compare related kinase activities, we analysed the UL97-homologous protein kinase BGLF4, encoded by EBV. BGLF4 exerted high levels of autophosphorylation (Fig. 3d) and GCV phosphorylation (Fig. 3c), the latter having not been demonstrated before. An important result was that neither of the two activities was sensitive to treatment with NGIC-I. The quantities of phosphorylation of GCV and BGLF4 autophosphorylation in NGIC-I-treated cells were unaltered compared with untreated cells (Fig. 3c, d). This stood in strict contrast with the significant inhibition of pUL97 assayed in parallel. This finding provides further evidence for the pUL97 specificity of indolocarbazole NGIC-I.

Deletion of UL97 leads to indolocarbazole-insensitivity

A virus deletion mutant, AD169delUL97, lacking most of the ORF-UL97 and being incapable of producing a functional viral pUL97 kinase, has been described previously (Prichard *et al.*, 1999). In our study, the lack of synthesis of pUL97 during virus replication in infected HFF cells was verified by indirect immunofluorescence (data not shown). The consequence of deletion of UL97, which has been described as a severe deficiency of virus replication *in vitro*, could be confirmed by our analysis (Fig. 4a). We further analysed this virus mutant for its replication characteristics in the presence of pUL97-specific indolocarbazoles or other antiviral drugs (Fig. 4b). CDV, targeting the viral DNA polymerase, had a significant inhibitory effect on the replication of both viruses, AD169 and AD169delUL97. This was measured as a reduced plaque formation (2% and 14%, respectively) in comparison with the untreated control (100%). NGIC-I, in contrast, did exclusively show an inhibitory effect on the replication of AD169 virus (30%), while no significant inhibition of AD169delUL97 virus could be detected (98%). This strongly argues for the pUL97 specificity of NGIC-I and, importantly, confirms the high potential provided by antiviral strategies targeting pUL97.

The antiviral activity of indolocarbazoles is selective for HCMV

The effect of indolocarbazoles was tested on a panel of viruses in the conventional plaque reduction assay (Fig. 4c). For each virus, the host cell type with high permissiveness for lytic virus replication was chosen. Treatment with either NGIC-I or Gö6976 (50 nM) led to a drastic reduction in the replication of two genetically different HCMVs, the laboratory strain AD169 and the endotheliotropic variant TB40E. We used the endotheliotropic variant to illustrate that different coding capacities of the viral genomes (Cha *et al.*, 1996; Prichard *et al.*, 2001) do not influence the sensitivity towards pUL97-targeting inhibitors. In contrast, a clinical isolate of HSV-1 was not inhibited by either of the two compounds. Furthermore, human influenza A virus (strain A/WSN/33) was also indolocarbazole-insensitive. Thus, the antiviral activity of NGIC-I and Gö6976 was selective for HCMV and was not detectable for other viruses such as HSV-1 or influenza A virus.

Discussion

Indolocarbazoles are attractive candidates for the development of a novel generation of antiviral drugs against HCMV. Our study provides the first direct evidence that selected indolocarbazole compounds are capable of targeting the viral kinase pUL97 in infected cells and thereby producing a strong inhibitory effect on HCMV replication. Four lines of evidence point to the conclusion that pUL97 is the primary target of the indolocarbazoles' antiviral action: (i) indolocarbazole-sensitivity is an invariable property of the pUL97 kinase activity, as it is detectable for wild-type pUL97 and GCV-resistant point mutants, but is not detectable for a related viral protein kinase (BGLF4); (ii) various strains of HCMV are indolocarbazole-sensitive and, moreover, GCV-resistant HCMV is hypersensitive; (iii) other viruses are found to be insensitive; and (iv) deletion of UL97 in the HCMV genome leads to a complete loss of indolocarbazole sensitivity. Thus, at least those indolocarbazole compounds that were selected for their high *in vitro* efficacy against pUL97 (NGIC-I and Gö6976) possess HCMV-inhibiting activity due to their ability to interfere with the viral protein kinase.

The chemical structure of glycosidic indolocarbazoles, such as the natural product STP from *Streptomyces staurosporeus*, can be divided into two units, the heterocyclic indolocarbazole core [H-indolo(2,3-a)pyrrolo(3,4-c)carbazole] and a carbohydrate moiety. Non-glycosidic indolocarbazole derivatives, such as NGIC-I, Gö6976 and Gö7874, have replaced the carbohydrate portion by variable non-glycosidic residues at the indolyl nitrogens (R1 and R2) (Kleinschroth *et al*., 1993; Pindur *et al*., 1999; Calbiochem product details). It is worth noting that the two compounds NGIC-I and Gö6976, which both possess high inhibitory capacities against pUL97 activity and HCMV replication, only differ by a single methyl residue at R1. On the other hand, compound Gö7874, which does not possess inhibitory activity, carries a larger substitution at R2 ($-CH_2CHOHCH_2NMe_2$) in additon to R1 methylation and further modifications. This might indicate that structural alterations, as introduced by substitution of small non-glycosidic residues at the indolyl nitrogens, largely determine the activity of indolocarbazole compounds with respect to the antiviral effect. The mechanism of indolocarbazole-mediated inhibition of serine/threonine protein kinases of the PKC family was described as the competitive blocking of the ATP binding site (reviewed by Goekjian & Jirousek, 1999). For pUL97, the structure of the ATP binding pocket has not yet been elucidated, so that a crystal structure model of pUL97, which might provide a basis for intensified structure–activity analysis, is highly desirable.

The specific role of pUL97 in the virus replication cycle has been focused by a recent publication (Wolf *et al*., 2001), demonstrating that pUL97 is involved in the regulation of both early and late steps of replication, and that the function of pUL97 increases the efficiency of viral DNA synthesis as well as capsid maturation. It was reported that the most decisive part of pUL97 activity might be directed towards viral capsid assembly, which was highly inefficient during infection with a UL97-deleted virus mutant. Given this background, it seemed probable that phosphorylation of more than one target protein resulted from pUL97 activity. This hypothesis was strengthened by the finding that various cellular proteins directly interacted with pUL97, as demonstrated by protein interaction studies (M. Marschall, unpublished results). In contrast to the frequent isolation of GCV-resistant viruses, no natural virus isolate encoding a catalytically inactive mutant of pUL97 has been detected so far. In particular, no variant of pUL97 lacking autophosphorylation could be identified. In detailed functional analyses of point and deletion mutants, autophosphorylation was always found as a prerequisite of other phosphorylation activities (Michel *et al*., 1998; P. Suchy & M. Marshall, unpublished results). Therefore, it seems suggestive that autophosphorylation of the pUL97 kinase is essentially connected with its active state and its natural role in virus replication.

pUL97, besides possessing autophosphorylation activity (Michel *et al*., 1998, 1999; He *et al*., 1997), efficiently phosphorylates common *in vitro* protein substrates, such as histone 2B (Marschall *et al*., 2000) and, *in vivo*, might be responsible for the hyperphosphorylation of translation factor EF-1δ (Kawaguchi *et al*., 1999). Furthermore, phosphorylation of pUL44, known as essential processivity factor of the viral DNA polymerase (Anders & McCue, 1996), was demonstrated *in vitro* (Krosky *et al*., 1999). This phosphorylation step may be based on a direct physical interaction between pUL97 and pUL44 (Marschall *et al*., 2001b; M. Marschall, unpublished results). However, the functional consequences of these phosphorylation activities remain speculative and await further investigation. Some parallels can be drawn from the characterization of the protein kinase BGLF4, encoded by EBV. BGLF4 is homologous to pUL97 and, *in vitro*, phosphorylates the pUL44-homologue BMRF1 (processivity factor of EBV DNA polymerase; Chen *et al*., 2000). Of note is the fact that this phosphorylation step is sensitive to inhibition by 1263W94 (Zacny *et al*., 1999). This finding might indicate that pUL97 and BGLF4 share functional similarities and that inhibitors of these protein kinases might block similar regulatory steps in the DNA synthesis of the two herpesviruses. Surprisingly, however, we found that the powerful inhibitor of pUL97 activity, NGIC-I, is completely ineffective in blocking BGLF4. Neither GCV phosphorylation by BGLF4, which has been demonstrated here for the first time and might explain conflicting reports on GCV insensitivity of EBV thymidine kinase (Gustafson *et al*., 1998), nor autophosphorylation is NGIC-I-sensitive. This strengthens the notion that NGIC-I exerts a high level of specificity for pUL97, but not for related herpesvirus protein kinases.

In this context it is particularly interesting that the replication of HSV-1 was insensitive to the two strongest HCMV-inhibiting indolocarbazoles, NGIC-I and Gö6976. This finding should be seen in the light of previous findings of Ng *et al*. (1996), demonstrating that pUL97 can partially substitute for its homologous protein kinase of HSV-1 (UL13). In cells infected with HSV-1 recombinants, in which UL13 was deleted and replaced by UL97 ($UL13^-$ $UL97^+$ virus), pUL97 mediated in part the post-translational modification of HSV-1 protein ICP22. This led to the conclusion that the mechanisms of substrate recognition of the two kinases were quite similar, at least in the case of the substrate ICP22. On the other hand, our data pointed to a major difference between the two kinases, concerning the indolocarbazole-mediated block of HCMV in contrast with the insensitivity of HSV-1. The finding indicates that, within the UL97 and UL13 proteins, binding sites for the indolocarbazoles may be structurally different. Such binding sites are postulated to lie in proximity to (or directly within) the ATP binding pockets of the kinase domains. In this respect, structural differences are likely to be explained by the limited homology between UL97 and UL13 (Chee *et al*., 1989). Thus, these findings underline the distinctness of protein substrate recognition and indolocarbazole-binding domains within the pUL97-related protein kinases.

In general, UL97 is a conserved gene, which is largely maintained in its wild-type sequence in the absence of the selective pressure of antiviral drugs such as GCV (Liu *et al.*, 2000). We undertook infection experiments to determine the formation of resistant HCMVs under the selective pressure of pUL97-specific inhibitors. Using low concentrations of NGIC-I or Gö6976 (starting with 10 nM and increasing stepwise to 100 nM) in infection experiments with AD169-GFP virus on HFFs, we could continuously monitor GFP expression and the growth of limited numbers of viral plaques, which were isolated and purified. This situation gave rise to the initial postulation that the selection of resistant viral clones was a frequent event. When these viruses were further passaged, however, the isolates were either lost (Gö6976) or were restricted to a low-level replication under antiviral treatment (NGIC-I), lacking the true phenotype of drug resistance. Furthermore, PCR amplification and sequence analysis of the UL97 gene of four of these isolates (NGIC-I) did not reveal any genotypic variation or resistance-conferring mutations (data not shown). The findings indicated that resistance formation towards these indolocarbazoles might not (or only rarely) occur under the applied conditions. This seemed not unexpected, considering the fact that indolocarbazoles are competitive inhibitors of ATP binding sites of kinases. Resistance-conferring mutations in these essential protein domains are likely to result in a kinase-defective phenotype, which might be detrimental for virus replication. Furthermore, we cannot rule out the possibility that other regulatory steps in virus replication, such as the recently detected kinase activities of other HCMV proteins, such as pp65 (Yao *et al.*, 2001), are targeted by indolocarbazoles. However, in the light of our observations, UL97 mutations may be sufficient but not necessary for resistance of HCMV towards indolocarbazoles. Further analysis of chemically different compounds of pUL97-directed virus inhibitors is under way and a larger set of data will be needed to a obtain closer insight into the mechanisms of resistance formation.

In general, these experiments have shown the high HCMV-specific antiviral potential of indolocarbazoles by a mechanism directly targeting the viral protein kinase. It will be challenging to continue studies on pUL97-specific inhibitors at pharmacological and clinical levels, and thus open new possibilities of HCMV therapy.

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