Indolocarbazoles exhibit strong antiviral activity against human cytomegalovirus and are potent inhibitors of the pUL97 protein kinase

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Abstract

We have analyzed a panel of protein kinase inhibitors (PKIs) and found that some indolocarbazoles (Gö6976, K252a, K252c) proved to be highly effective inhibitors of GCV-sensitive and -resistant human cytomegalovirus (HCMV) strains, but did not show any effect against herpes simplex virus. Antiviral activity was determined by focus reduction assays (IC_{50} ranging from 0.009 to 0.4 μM). Other inhibitors of serine/threonine kinases (Gö6850, H-7, roscovitine) were found to be ineffective. Virus yield at 5 days after infection was reduced by three orders of magnitude with nanomolar concentrations of the indolocarbazoles. These compounds were fully effective when added up to 24 h post infection and showed reduced activity up to 72 h post infection. Cytotoxicity assays in proliferating and non-proliferating cells demonstrated that the effective antiviral concentration of these compounds was significantly lower than either antiproliferative (IC_{50}/CC_{50} ranging from 6.5 to 390) or cytotoxic (IC_{50}/CC_{50} ranging from 72.5 to 1000) doses. The effects of PKIs on the virus-encoded protein kinase pUL97 were studied using recombinant vaccinia viruses. Indolocarbazoles strongly inhibited both pUL97 autophosphorylation (IC_{50} ranging from 0.0012 to 0.013 μM) and pUL97-dependent ganciclovir phosphorylation (IC_{50} ranging from 0.05 to 0.26 μM). Other inhibitors of serine/threonine kinases showed only weak (Gö6850) or no (H-7, roscovitine) effect on these pUL97 functions, while oxoflavone tyrosine kinase inhibitors had no effect at all. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Human cytomegalovirus; Antiviral activity; Protein kinase; Indolocarbazole

1. Introduction

For treatment of human cytomegalovirus (HCMV) disease the antiviral drugs ganciclovir (GCV), foscarnet (PFA) and cidofovir (HPMPC) are approved. Prolonged or repeated GCV therapy for treatment of CMV disease predisposes for

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the selection of resistant viruses. Especially in highly immunosuppressed patients who suffer from a high HCMV virus load, the emergence of ganciclovir-resistant HCMV seems to be relatively common and is associated with progressive CMV disease (Erice et al., 1989). Hence, problems arising from the development of drug-resistant strains (Chou et al., 1997; Erice et al., 1998) have been reported. The drugs licensed so far for HCMV chemotherapy are all targeted to the viral DNA polymerase. There is urgent need for the development of drugs with alternative mechanisms of action and/or directed to new targets.

Protein phosphorylation appears to be a key event in the HCMV life cycle, since a increasing number of HCMV-encoded phosphoproteins has been described (van Zeijl et al., 1997; Li et al., 1999; Zini et al., 1999) and inhibition of protein phosphorylation has been shown to interfere with HCMV replication (Bresnahan et al., 1997; Slater et al., 1999). Additionally, HCMV itself encodes for gene products which have been involved in protein kinase activity. He et al. (1997) demonstrated that pUL97 has the capability to autophosphorylate at serine and threonine residues, and the homologous gene products of herpes simplex virus (UL13) and varicella-zoster virus (ORF 47) have been shown to effect phosphorylation of regulatory proteins (Purves and Roizman, 1992; Ng et al., 1994). pUL97 can partially substitute for the function of the HSV 1 UL13 protein kinase and is able to phosphorylate the HSV alpha 22 protein (Ng et al., 1996) and possibly the cellular elongation factor EF-1δ (Kawaguchi et al., 1999). Furthermore, we could demonstrate that autophosphorylation of pUL97 is a prerequisite for GCV phosphorylation (Michel et al., 1999) whereas naturally occurring UL97 mutations in virus isolates from GCV treated patients result in impaired GCV phosphorylation but do not interfere with phosphorylation of pUL97 (Michel et al., 1998). Recently, it was reported that a recombinant HCMV containing a large deletion in the UL97 ORF exhibited a severe replication deficiency (Prichard et al., 1999). Besides pUL97 HCMV encodes further gene products which have been involved in protein kinase activity. The IE1-p72 gene product is able to

autophosphorylate serine residues, and phosphorylates E2F transcription factor and pocket proteins (Pajovic et al., 1997). Protein kinase activity has also been associated with the virus particle (Michelson et al., 1985; Britt and Auger, 1986; Roby and Gibson, 1986). The role of this virion-associated kinase activity has not been unravelled so far, although a participation of the tegument protein pp65 has been discussed (Schmolke et al., 1995).

Taken together, there is growing evidence that inhibition of virus-directed protein phosphorylation might be a useful target for anti-HCMV chemotherapy. Here we report the analysis of a panel of protein kinase inhibitors (PKIs) with respect to their antiviral activity against HCMV and with respect to their inhibitory potency on the virus-encoded protein kinase pUL97.

2. Materials and methods

2.1. Cells and viruses

Experiments with HCMV strains AD169, A6245 and HCMV-6 were performed using human foreskin fibroblasts (HFF) or human embryonic lung cells (HEL) as described previously (Michel et al., 1996). A6245 was isolated from a bone marrow transplant patient in our laboratory. HCMV-6 was a kind gift from A. Erice, University of Minnesota Medical School, MS (Hanson et al., 1995). CV1 or 143B TK-deficient cells were used for infection with recombinant vaccinia viruses (rVV). Construction and characterisation of rVV has been reported previously (Michel et al., 1998). HSV strain K1 was grown and titered on Vero cells, as described previously (Zimmermann et al., 1997).

2.2. Antiviral compounds

Ganciclovir (Cymevene®) was purchased from Syntex, Aachen, Germany, and roscovitine from Calbiochem, Bad Soden, Germany. All other protein kinase inhibitors were purchased from Alexis, Grünberg, Germany. Stock solutions of PKIs were prepared in DMSO at a final concentration of 10 mM and stored at −20°C.

2.3. Analysis of drug susceptibility

Susceptibility of HCMV strains to inhibition by PKIs was tested by focus reduction assay, and the effective dose for each antiviral compound was expressed as IC_{50} representing the inhibitory concentration of drug (μM) which reduced the number of viral foci by 50% exactly as described previously (Zimmermann et al., 1997). Staining of HCMV-infected cells was performed according to Gleaves et al. (1987). The sensitivity of the HSV1 strain to inhibition by PKIs was tested in a quantitative cytopathic effect (CPE) reduction assay exactly as described by Kruppenbacher et al. (1994). For determination of HCMV virus yield HEL cells were infected with HCMV AD169 at an MOI of 1. One day p.i. the supernatant was replaced by fresh MEM. At 5 days p.i. the supernatant of infected cells was removed and centrifuged for 30 min at $3000 \times g$. In order to pellet the virus particles the clarified supernatant was subjected to ultracentrifugation at $80\,000 \times g$ for 2 h. Virus was resuspended in MEM and titrated by fivefold serial dilution steps on HEL cells. The number of infected cells was determined by immunostaining as described above.

2.4. Analysis of cell viability and proliferation

The cytotoxicity of PKIs was determined by using a neutral red based cytotoxicity assay. Briefly, serial two-fold dilutions of the respective drugs were prepared in MEM and 100 μl of the diluted drug were added to confluent HEL cells in a 96-well microtiter plate. After incubating the plates for 5 days at 37°C in presence of drug, the MEM was removed, the cells were washed with 400 μl PBS and incubated for 3 h with 100 μl of 0.1% neutral red in PBS. The dye solution was removed and the cells were washed again with 400 μl of PBS. In order to extract the dye 200 μl of a solution containing 50% methanol and 1% acetic acid was added and incubated for 15 min at room temperature. The OD of the neutral red dye was determined using an ELISA reader at a wavelength of 550 nm and a reference wavelength of 690 nm. The CC_{50} was calculated by regression analysis. For analysis of cell proliferation cells were seeded at a density of 2×10^3 cells/well. The cells were allowed to adhere and the number of viable cells was determined exactly as described above.

2.5. Analysis of ganciclovir anabolism

[8-^{3}H]-ganciclovir (specific activity: 13.2 Ci/mmol) was used for analysis of nucleoside anabolism. Infection of 143B cells with rVV, extraction of nucleosides and HPLC analysis were done exactly as described previously (Zimmermann et al., 1997). GCV phosphorylation was determined in duplicate from untreated controls and from cells treated with serial two-fold dilutions of drug.

2.6. Analysis of protein phosphorylation

pUL97 phosphorylating activity was analyzed from nuclear fractions of CV1 cells infected with rVV231 expressing a functional pUL97 and cell fractionation was performed essentially as described previously (Michel et al., 1998). The final pellet was reextracted in 10 mM Tris/HCl, pH 7.5, 1 M NaCl, 20 mM DTT, 0.2 mM MgCl$_2$, 0.2 mM EGTA, 0.1 mM PMSF. The supernatant of this extraction step was used for in vitro kinase assays. pUL97 autophosphorylation was analyzed in a reaction buffer containing 50 mM Tris/HCl pH 9.0, 500 mM NaCl, 10 mM MgCl$_2$, 5 mM DTT, 5 μM ATP and 2,5 μCi [γ-^{33}P] ATP/200 μl (specific activity $\geq$ 6000 Ci/mmol). After incubation at 37°C for 30 min the reaction was terminated by adding 50 μl of 50% trichloroacetic acid. Total protein was precipitated by centrifugation at $14\,000 \times g$ for 15 min at 4°C. The protein pellet was washed with 100% ethanol, resuspended in Laemmli sample buffer and boiled for 5 min. The phosphorylated proteins were separated by SDS-PAGE (12% acrylamide) and detected by autoradiography. The quantitative analysis of protein phosphorylation was performed using a Phospho-Imager and ImageQuant software (both by Molecular Dynamics, Krefeld, Germany). The particle associated kinase activity was determined
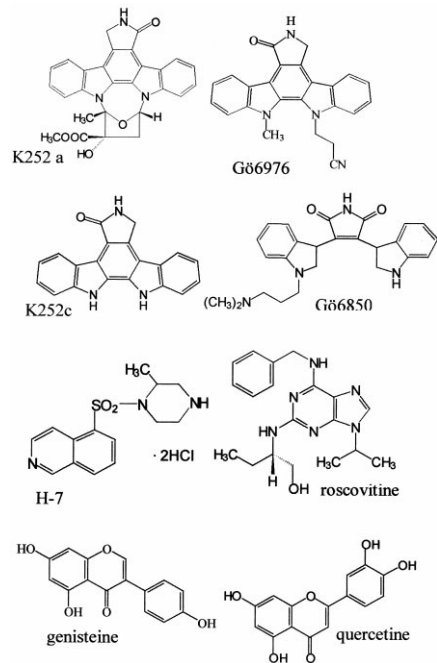
Fig. 1. Structural formulae of protein kinase inhibitors (PKIs).

3. Results

3.1. Antiviral activity of protein kinase inhibitors

We have investigated different protein kinase inhibitors (PKIs) with respect to their antiviral effect against HCMV. The structures of the PKIs used are shown in Fig. 1. The antiviral activity of PKI was tested by focus reduction assays using three HCMV strains. The results are listed in Table 1. The indolocarbazole derivatives K252a, K252c and Gö6976 exhibited a clear antiviral effect against the HCMV laboratory strain AD169 with IC_{50} values in the nanomolar range, which was markedly lower than the IC_{50} of GCV. In contrast, other inhibitors of serine/threonine kinases, Gö6850 (bisindoylmaleimide I), the isoquinolonesulfonamide H-7 and roscovitine, which is a strong inhibitor of the cyclin dependent kinase cdk2a, showed a much weaker antiviral activity with IC_{50} values in the micromolar range. Likewise, oxoflavone inhibitors of tyrosine kinases (genisteine, quercetine) provided only weak antiviral activity. Gö6976, K252a and K252c also proved to be effective against an HCMV patient isolate (A6245) and a GCV-resistant strain (HCMV-6), which contains the amino acid exchange H520-Q (Hanson et al., 1995). The IC_{50} values for these strains showed only minor differences within a two-fold range as compared to the

exactly as described by Mar et al. (1981). Protein phosphorylation was determined either without drug or in the presence of twofold serial dilutions of drug.

Table 1
Antiviral activity of protein kinase inhibitors against HCMV and HSV1 in comparison with GCV

Compound	IC_{50} (µM)[a]			
	HCMV AD169	HCMV A6245	HCMV 6	HSV1 K1
K252a	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.005	> 25
K252c	0.32 ± 0.22	0.17 ± 0.12	0.13 ± 0.08	> 25
Gö6976	0.04 ± 0.01	0.05 ± 0.01	0.009 ± 0.002	> 25
Gö6850	9.5 ± 0.8	12.5 ± 1.0	6.4 ± 0.5	n.d.[b]
H-7	12.5 ± 2.9	22.5 ± 1.5	23.6 ± 1.8	n.d.[b]
Roscovitine	13.5 ± 1.5	13.5 ± 1.5	13.5 ± 1.5	n.d.[b]
Genisteine	31.1 ± 1.9	32.4 ± 2.9	26.7 ± 1.5	n.d.[b]
Quercetine	10.1 ± 2.5	12.5 ± 2.5	8.4 ± 1.2	n.d.[b]
GCV	3.8 ± 0.8	1.8 ± 0.8	42.7 ± 5.4	0.1 ± 0.05

[a] IC_{50}, the concentration of drug required to reduce focus formation (HCMV) or cytopathic effect (HSV) by 50%; each value represents the mean $\pm$ S.D. from at least three separate experiments.
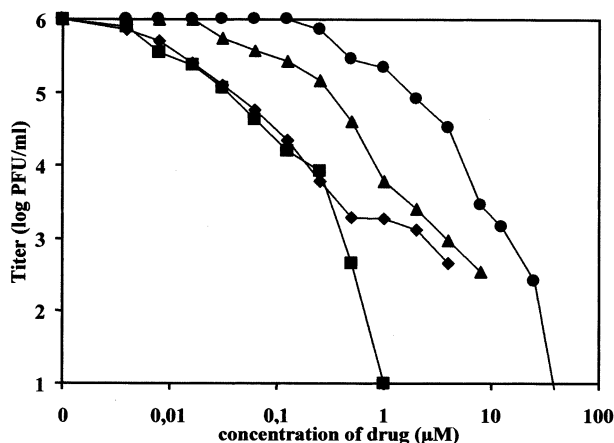[b] n.d., not determined.

Fig. 2. Dose-dependent reduction of HCMV virus yield by indolcarbozoles in comparison to GCV. HFF were infected with HCMV AD169 at an MOI of 1 PFU/cell and treated with the indicated concentrations of Gö6976 (◆), K252a (■), K252c (▲), and GCV (●). At 5 days post infection, the supernatant was collected and the virus titer was determined by titration. Virus titers are shown in log units PFU per ml. Data at 0 μm represent the titer derived from controls treated with DMSO at the concentration used for the corresponding drug dilution. All values represent the mean from at least three independent experiments.

Table 2
Effect of protein kinase inhibitors on viability and proliferation of HEL cells

Compound	CC_{50} (μM)[a]	
	Confluent HEL	Proliferating HEL
K252a	2.9 ± 1.0	0.25 ± 0.04
K252c	29.3 ± 10.6	8.0 ± 6.0
Gö6976	>40	15.6 ± 8.0
Staurosporine	<0.01	<0.01
Gö6850	>40	>40
H-7	16.1 ± 2.7	6.6 ± 4.5
Roscovitine	32.5 ± 12.5	15.0 ± 6.5
Genisteine	90.0 ± 14.0	19.6 ± 0.6
Quercetine	131.8 ± 45.0	73.2 ± 26.8

[a] CC_{50}, the concentration of drug required to reduce the neutral red uptake of cells by 50%; each value represents the mean ± S.D. from at least three separate experiments.

respective values for AD169. In order to test the virus selectivity of the effective indolocarbazoles we also performed inhibition tests with herpes simplex virus. However, no antiviral effect could

be observed (Table 1). These data indicate that Gö6976, K252a and K252c are potent and specific inhibitors of HCMV replication. In another series of experiments we studied the antiviral activity of indolocarbazoles by determination of the virus yield reduction under drug. Gö6976 and K252c reduced the virus yield from cell cultures infected at an MOI of one or higher in a dose-dependent manner by at least three orders of magnitude (Fig. 2) but did not completely abolish virus replication. Only K252a provided a complete loss of virus yield at concentrations > 500 nM. No virus yield reduction was achieved using the other PKIs

3.2. Effect of protein kinase inhibitors on cell viability and cell proliferation

We studied the cytotoxic effects of PKIs by determination of neutral red uptake by viable cells after incubation of confluent layers of HEL cells for 5 days with the respective inhibitor. The PKIs used in this study showed a cytotoxicity ranging from 2.9 to 131.8 μM (Table 2). In contrast, the indolocarbazole reference compound staurosporine showed a dramatic cytotoxic effect at a concentration as low as 10 nM.

Additionally, we determined the inhibitory effects of PKIs on proliferating HEL cells. For this purpose, HEL cells were seeded at low density (2000 cells/0.28 cm²-well) and allowed to proliferate in presence of PKIs for 5 days. The results are also summarized in Table 2, indicating a more pronounced antiproliferative effect of PKIs as compared to cytotoxicity. With the indolocarbazoles Gö6976 and K252c the CC_{50} for confluent as well as proliferating cells was 20–200-fold higher than the corresponding doses required for inhibition of HCMV replication indicating a reasonable therapeutic index for these compounds. For K252a, the observed cytotoxicity was also moderate, however, K252a exhibited a pronounced antiproliferative effect at doses > 500 nM. This result might also explain the complete abrogation of HCMV replication observed at the high concentration range. Since it has already been shown that the inhibition of cdk2 by roscovitine resulted in a complete inhibition of HCMV

replication (Bresnahan et al., 1997), it seems reasonable to assume that the effect of K252a is at least partially induced by interaction with cellular kinases resulting in a cell cycle arrest in treated cells.

3.3. Effect of addition after different times post infection on the antiviral activity of indolocarbazoles

In order to get first insight at which time point of the HCMV replication cycle the indolocarbazoles exert their antiviral effect in comparison to GCV, we added the compounds at different time points after infection. Five days after infection the supernatants from the infected cell cultures were titrated on HEL and the virus yield was determined. As outlined in Table 3, the addition of indolocarbazoles to infected cells can be delayed for 24 h without significant loss of antiviral activity; thereafter, the compounds progressively lost their antiviral effect. However, up to 72 h a weaker antiviral activity resulting in a three- to five-fold reduction of virus yield could be reproducibly observed. The ten-fold virus yield reduction obtained with K252a might at least partially be explained by cumulative effects with respect to cellular kinases as outlined above. A loss of antiviral activity when treatment was delayed in the replication cycle of HCMV was also observed in infected cell cultures treated with GCV. Only a marginal virus yield reduction up to two-fold

could be achieved if the compound was added at 72 h post infection. Therefore, in contrast to GCV, the indolocarbazoles are active even at late time points of the HCMV replication cycle. These data indicate that the mechanism of action of these compounds is most probably not based on the inhibition of the viral DNA polymerase and therefore is different from the action of GCV.

3.4. Effect of protein kinase inhibitors on the HCMV-encoded protein kinase pUL97

Since HCMV encodes for the functional protein kinase pUL97, we have investigated the effect of PKIs on this enzyme. We showed before that the expression of the human cytomegalovirus UL97 protein in recombinant vaccinia viruses (rVV) is a suitable system for studying all known pUL97 functions such as pUL97-dependent autophosphorylation and pUL97-dependent GCV phosphorylation (Michel et al., 1998). Furthermore, the use of rVV allows the analysis of pUL97 functions in the absence of other HCMV gene products. In order to determine the intracellular inhibition of pUL97 functions by PKIs we investigated the effects of different PKIs on pUL97-dependent GCV phosphorylation in cells infected by rVV. The pUL97-dependent GCV phosphorylation was strongly inhibited by all indolocarbazoles tested (Gö6976, K252a, K252c), while bisindoylmaleimide I (Gö6850), an other inhibitor of serine/threonine kinases, showed a much weaker

Table 3
Effects of indolocarbazoles and GCV on HCMV virus yield when added at different times after infection

Time of drug addition post infection (h)[a]	Titer (PFU/ml)[b]			
	Gö6976[c]	K252a[c]	K252c[c]	GCV[c]
0	3×10^2	4×10^2	4×10^3	< 100
2	4×10^2	6×10^2	8×10^3	<100
24	6×10^2	1.5×10^3	2×10^4	1×10^2
48	8×10^3	1.5×10^4	5×10^4	8×10^3
72	1.5×10^5	6×10^4	2×10^5	6×10^5
Control	8×10^5			

[a] Time points were calculated from the time of virus inoculation; drugs at time 0 were added simultaneously with the inoculum.
[b] HCMV (strain AD169) was titrated on HEL cells at 5 days post infection; infected cells treated with the highest concentration of DMSO needed for drug dilution served as control.
[c] Gö6976 and K252c were used at a concentration of 0.5 μM, K252a and GCV at 0.25 and 25 μM, respectively.

Table 4

Inhibitory effect of protein kinase inhibitors on pUL97-dependent ganciclovir phosphorylation and pUL97 autophosphorylation

Compound	IC$_{50}$ (μM) of the respective activity[a]	
	pUL97-dependent GCV phosphorylation[b]	pUL97 autophosphorylation[c]
K252a	0.026 ± 0.003	0.001 ± 0.001
K252c	0.025 ± 0.007	0.009 ± 0.003
Gö6976	0.005 ± 0.001	0.004 ± 0.002
Staurosporine	n.d.[d]	0.013 ± 0.003
Gö6850	4.5 ± 1.5	0.79 ± 0.09
H-7	> 5	> 10
Roscovitine	> 5	> 10
Genisteine	> 5	> 10
Quercetine	> 5	> 10

[a] Each value represents the mean ± S.D from at least three separate experiments.

[b] The concentration of drug required to reduce the amount of phosphorylated GCV in rVV231 infected cells as quantified by HPLC analysis by 50%.

[c] The concentration of drug required to reduce phosphorylation of pUL97 by 50%, as determined by in vitro kinase assay and quantified by phosphoimaging.

[d] n.d., not determined due to cytotoxicity of the compound.

inhibitory effect. No inhibition of GCV phosphorylation could be achieved by other PKIs. The IC$_{50}$ values calculated for the inhibition of pUL97 dependent GCV phosphorylation by PKIs are summarized in Table 4. The inhibition of pUL97-dependent GCV phosphorylation by PKIs was strongly dose-dependent (Fig. 3(A)). In order to rule out any effect of PKIs on the quantitative expression of pUL97 in this assay, each inhibition experiment was accompanied by western blot analysis for monitoring pUL97 expression. Using the indolocarbazoles Gö6976, K252a and K252c, no influence on pUL97 expression was detected (Fig. 3(B)). Interestingly, increasing concentrations of indolocarbazoles resulted in changes of the electrophoretic mobility of pUL97 and finally in the appearance of a slightly faster migrating pUL97-band. These results are in line with data reported by Michel et al. (1999), who observed similar differences in the pUL97 electrophoretic pattern when expressing UL97 mutants which are autophosphorylation-deficient. Additionally, van

Zeijl et al. (1997) have demonstrated that the dephosphorylated form of pUL97 migrates slightly faster in SDS gels than phosphorylated pUL97. In conclusion, we presume that the dose-dependent differences in the electrophoretic pattern of pUL97 may reflect an intracellular inhibition of pUL97 autophosphorylation. In consequence, we investigated the effects of PKIs on pUL97 autophosphorylation in vitro. The inhibition of pUL97 autophosphorylation was determined quantitatively by phosphoimaging, and the effects of PKIs on pUL97 autophosphorylation are also summarized in Table 4. Similarly as for the pUL97-dependent GCV phosphorylation, all indolocarbazoles tested (Gö6976, K252a, K252c) were strong inhibitors of pUL97 autophosphorylation in a dose-dependent manner. However, it should be noted that the inhibition of pUL97 autophosphorylation in vitro appeared much stronger than should be expected from the differences in electrophoretic mobility described above. Hence, we cannot rule out that there exist other factors which influence the intracellular interaction of PKIs and pUL97.

Aside from the indolocarbazoles only the bisindoylmaleimide Gö6850 showed a weak inhibition of pUL97 autophosphorylation while all other PKIs failed to inhibit this function. Since PKIs which do not contain the indolocarbazole structure were not able to inhibit pUL97, we presume that the interaction of the indolocarbazole structure with pUL97 is most probably responsible for the inhibition of the enzyme. Hence, we additionally analyzed the indolocarbazole compound staurosporine which could not be tested in cell culture assays due to its pronounced cytotoxicity and found that the inhibition of pUL97 autophosphorylation by staurosporine was comparable to the inhibition by other indolocarbazoles.

3.5. Effect of protein kinase inhibitors on HCMV particle-associated kinase activity

Protein kinase activity has also been associated with the virus particle (Michelson et al., 1985; Britt and Auger, 1986; Roby and Gibson, 1986). The role of these virion-associated kinase activi-

ties has not been unravelled yet although a participation of the tegument protein pp65 has been discussed (Schmolke et al., 1995). We analyzed the effects of PKIs on the particle associated protein phosphorylation and found that indolocarbazoles had only minor effects on this activity with inhibitory doses in the micromolar range (data not shown). In our hands quercetine appeared to be the most potent inhibitor of the virion associated kinase activity as has been described by others (Michelson et al., 1985).

3.6. Antagonism of protein kinase inhibitors and ganciclovir

Since the indolocarbazoles Gö6976 and K252c were found to be strong inhibitors of pUL97-dependent GCV phosphorylation using the vaccinia virus expression system, we hypothesized that these PKIs might antagonize the effect of GCV. Hence, we performed virus yield reduction experiments in which both, an indolocarbazole and GCV, were added. Based on the mechanism of GCV activation via phosphorylation by pUL97 we expected that addition of an indolocarbazole

to cells incubated with GCV should at least partially counteract the effect of GCV. This should result in an increase of virus yield at concentrations of indolocarbazole sufficient to inhibit GCV phosphorylation, as determined in our vaccinia virus system, but not high enough for suppression of HCMV replication. Indeed, we found a tenfold increased virus yield comparing cells treated with 10 or 50 µM GCV in combination with 100 nM Gö6976 and cells treated with GCV alone (Fig. 4). This observation indicates that Gö6976 was at least partially able to antagonize the antiviral activity of GCV. When using higher concentrations of Gö6976 the antagonistic effect was less pronounced due to the virus yield reduction achieved by Gö6976 itself. Similar results were also obtained using K252a and K252c (data not shown).

4. Discussion

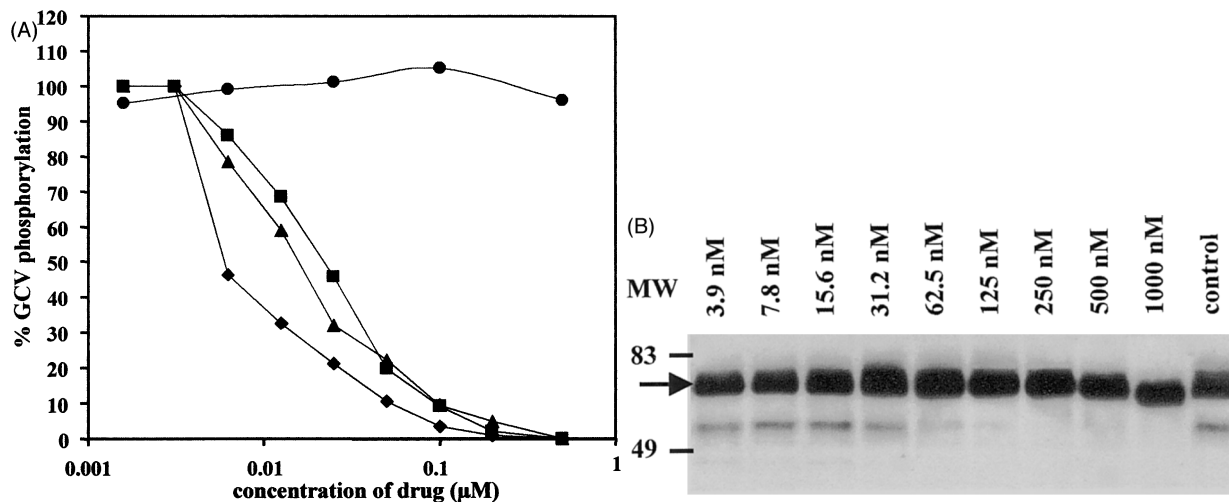We have analyzed the antiviral activity of protein kinase inhibitors against HCMV and

Fig. 3. (A) Dose-dependent inhibition of pUL97-dependant GCV phosphorylation by Gö6976 (♦), K252a (■), K252c (▲) in comparison to roscovitine (●). Cells were infected with rVV231 and harvested at 20 h post-infection. GCV-phosphates were extracted from the cells and quantified by HPLC. The values presented are expressed as a percentage of the untreated control. Each value is derived from at least three independent experiments. (B) Expression of pUL97 in cells infected with rVV231 (control) and treated with the indicated amounts of Gö6976. Total protein was extracted from an aliquot of the same cells used for the determination of pUL97-dependent GCV phosphorylation. Equal quantities of the cell lysates were separated by 12% SDS-PAGE , and the proteins were visualized by chemoluminescence in a Western Blot analysis with a monoclonal pUL97 antibody.
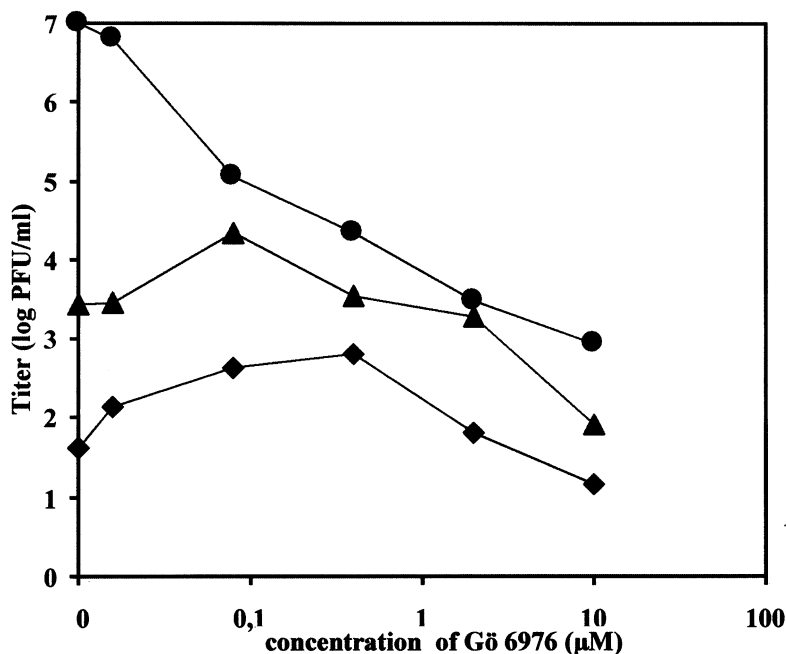
Fig. 4. Antagonism of GCV and Gö6976 in HFF infected by HCMV AD169. HFF were infected with HCMV AD169 at an MOI of 1 PFU/cell and treated with the indicated concentrations of Gö6976 in combination with 0 (●), 10 (▲) and 50 (♦) μM GCV. At 5 days post infection, the supernatant was collected and the virus titer was determined by back titration. Virus titers are shown in log units PFU per ml. Each value is derived from three independent experiments.

found that one class of PKIs, the indolocarbazoles, were potent inhibitors of HCMV replication but did not influence replication of HSV 1. A comparison of the activities of indolocarbazoles and GCV revealed that the indolocarbazoles exhibited a prolonged activity during the HCMV replication cycle and that action of indolocarbazoles was antagonistic to the action of GCV. Taken together, these data indicate that the mechanism of action of the indolocarbazoles Gö6976 and K252c is different from the mechanism of GCV action. Hence, the indolocarbazoles may represent a new class of inhibitors of HCMV replication. Mechanistically, they are quite different from the drugs licensed so far for HCMV chemotherapy which are all targeted to the viral DNA polymerase. Additionally, we have shown in this paper that the indolocarbazoles are also effective against a GCV-resistant HCMV strain.

The HCMV pUL97 has been characterized as a protein kinase which autophosphorylates and is capable to phosphorylate ganciclovir. Addition-

ally, there is growing evidence that the function of pUL97 is important for HCMV replication. We have analyzed the influence of indolocarbazoles on these activities and found them to be strong inhibitors of pUL97 autophosphorylation as well as ganciclovir phosphorylation in cells infected with recombinant vaccinia viruses expressing a functionally active pUL97. These data provide further evidence that pUL97 autophosphorylation is indeed a prerequisite for GCV phosphorylation as described earlier (Michel et al., 1999).

The inhibitors used are capable to interact with the ATP binding site of Ser/Thr kinases characterised by the G–X–G–X–X–G motif located in domain II (Chee et al., 1989; Michel et al., 1999). The strong inhibition of pUL97 functions by these compounds underlines that this ATP binding site may be essential for pUL97 function. The natural substrates of pUL97 are still unknown, and the inhibitors described here might also be helpful to investigate potential viral or cellular substrates of pUL97. The influence of indolocar-

bazoles on protein phosphorylation during HCMV infection is currently under investigation. There is an obvious correlation between the potency of PKIs to inhibit pUL97 functions and their antiviral effects against HCMV. Additionally, the quantitative reduction of virus yield obtained by the antiviral action of the PKIs Gö6976 and K252c is comparable to the replication deficiency which has been observed for the very recently described UL97-deficient HCMV (Prichard et al., 1999). Hence, the pUL97 protein kinase appears to be a possible molecular target of the indolocarbazoles. Experiments are currently under way to select PKI-resistant HCMV in vitro. Molecular characterization of these viruses and, finally, the construction of recombinant HCMV will provide direct proof for a possible involvement of pUL97. If so, the indolocarbazole compounds described represent lead compounds in order to create even more selective inhibitors of the pUL97 kinase by molecular modelling. Since all PKIs discussed here interact with the nucleotide binding site of the kinase a precise knowledge of the protein substrates of the viral kinase would allow the design of substrate analogues.

Besides pUL97, HCMV encodes further gene products which have been involved in protein kinase activity. We have investigated the effects of PKIs on the particle associated kinase activity but did not find evidence that an inhibition of this function might be involved in the inhibition of HCMV replication by indolocarbazoles. However, at the moment we cannot rule out the possibility that other virus-encoded enzymes which require ATP are involved in the mechanism of action of PKIs.

Apart from effects on virus encoded kinases, it has to be taken into account that cellular Ser/Thr kinases might be involved in the antiviral effect of PKIs against HCMV. One class of Ser/Thr kinases which possibly could cause an inhibition of HCMV replication are kinases involved in cell cycle regulation. HCMV is capable to interfere with cell cycle progression at multiple points (Bresnahan et al., 1996; Lu and Shenk, 1996, 1999). Recently, a direct interaction of the HCMV protein pp65 with the polo-like kinase plk1 has been demonstrated (Gallina et al., 1999). plk1 is a Ser/Thr kinase involved in the assembly of the mitotic spindle and in the control of the G2/M boundary of the cell cycle (Golsteyn et al., 1995; Hamanaka et al., 1995; Uchiumi et al., 1997), and therefore represents a new cellular enzyme which function might be essential for HCMV replication. A direct connection between inhibition of a cellular protein kinase and of HCMV replication has been demonstrated by blocking HCMV replication using the cdk2 inhibitor roscovitine (Bresnahan et al., 1997). However, it seems reasonable to assume that an antiviral action by inhibition of cellular kinases interfering with cell cycle regulation is accompanied by an antiproliferative effect of the respective drug. The cytotoxicity data presented in Table 2 for proliferating cells are in line with this presumption since a pronounced antiviral effect is observed with all PKIs tested (including roscovitine) when used at antiproliferative concentrations. Exceptions are the indolocarbazoles Gö6976 and K252c which exhibit their antiviral action in the nontoxic nanomolar range. Hence, we presume that inhibition of cellular kinases involved in cell cycle regulation might interfere with HCMV replication.

Another class of cellular Ser/Thr kinases are the signal transducing kinases including protein kinase C (PKC) and cAMP-dependent protein kinase (PKA). Interactions of PKC function and viral replication have been described for HSV 1 (Castagnino et al., 1995), Epstein-Barr-Virus (Cirone et al., 1990) and other enveloped viruses (Constantinescu et al., 1991). For HCMV it has been reported that infection of endothelial cells might be PKC-dependent (Slobbe van Drunen et al., 1997). The effect of PKIs on signal transducing kinases has been studied extensively and inhibitory concentrations of most PKIs to these kinases are available (Hashimoto et al., 1991; Fabre et al., 1993; Qatsha et al., 1993; Gekeler et al., 1996). However, no correlation between the inhibition patterns of signal transducing kinases and the antiviral activity of PKIs could be found. Thus, inhibition of signal transducing kinases may not be responsible for the anti-HCMV effect of PKIs. In conclusion, the indolocarbazole PKIs might represent lead substances for the development of better compounds for the inhibition of

HCMV. However, further research is needed in order to elucidate the precise mechanism of action of these compounds.

Acknowledgements

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