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Identification of Inhibitors for a Virally Encoded Protein Kinase by 2 Different Screening Systems: In Vitro Kinase Assay and In-Cell Activity Assay

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The human cytomegalovirus (HCMV) protein kinase pUL97 represents an important determinant for viral replication and thus is a promising target for the treatment of HCMV. The authors screened a compound library of nearly 5000 entities based on known kinase inhibitors in 2 distinct ways. A radioactive in vitro kinase assay was performed with recombinant pUL97, purified from baculovirus-infected insect cells, on myelin basic protein-coated FlashPlates. About 20% of all compounds tested inhibited pUL97 kinase activity by more than 50% at a concentration of 10 µM. These hits belonged to various structural classes. To elucidate their potential to inhibit pUL97 in a cellular context, all compounds of the library were also tested in a cell-based activity assay. For this reason, a HEK293 cell line was established that ectopically expressed pUL97. When these cells were incubated with ganciclovir (GCV), pUL97 phosphorylated GCV to its monophosphate, which subsequently became phosphorylated to cytotoxic metabolites by cellular enzymes. Thereby, pUL97 converted cells into a GCV-sensitive phenotype. Inhibition of the pUL97 kinase activity resulted in protection of the cells against the cytotoxic effects of GCV. In total, 199 compounds of the library were cellular active at nontoxic concentrations, and 93 of them inhibited pUL97 in the in vitro kinase assay. Among these, promising inhibitors of HCMV replication were identified. The 2-fold screening system described here should facilitate the development of pUL97 inhibitors into potent drug candidates. (Journal of Biomolecular Screening 2005:36-45)

Key words: kinase, human cytomegalovirus, cellular assay, drug discovery, pUL97

INTRODUCTION

HUMAN CYTOMEGALOVIRUS (HCMV) IS A MEMBER of the sub-group of beta-herpesviruses. HCMV seroprevalence rates in adults vary between 45% and 85% in the Western world. In most individuals with intact immune systems, HCMV causes little or no apparent clinical symptoms. However, in immunocompromised persons, HCMV can lead to serious diseases or mortality.1 Prior to the availability of a potent therapy against human immunodeficiency virus (HIV), HCMV-associated retinitis was commonly seen in HIV-positive patients.2 Patients who are immunosuppressed following bone marrow or solid organ transplantation remain at high risk of HCMV infection. In these patients, HCMV can lead to severe conditions such as pneumonitis and colitis or to complications such as acute or chronic rejection of a transplanted organ.3 Unborn and newborn children are another group at risk, and about 1% of babies have congenital HCMV infection, which may cause deafness or even death.4

Present standard treatments (e.g., with ganciclovir [GCV]) have the disadvantage of resistance formation. Furthermore, they cannot be used to treat HCMV-infected infants because of severe side effects (reviewed in Crumpacker5). Therefore, a strong demand exists for drugs with an improved safety profile.

To open new avenues in treating HCMV patients, we focused on the development of inhibitors directed against a viral protein kinase as a novel target. The HCMV gene UL97 codes for a kinase that phosphorylates nucleosides and proteins on serine and/or threonine residues, as well as performs autophosphorylation.1,6-10 pUL97 has been found to be responsible for the antiviral and
cytotoxic potency of GCV and related nucleoside analogs: it phosphor ylates the purine nucleoside analog to its monophosphate, which subsequently becomes further phosphorylated by cellular enzymes involved in nucleotide metabolism. The GCV triphosphate inhibits viral DNA synthesis in several ways: (1) inhibition of the viral DNA polymerase by competition with the natural nucleoside triphosphate (dTTP) and (2) chain termination of growing DNA. The latter aspect is the reason that repair and replication of cellular DNA are also affected by phosphorylated GCV, thus causing cytotoxicity.

pUL97 is primarily localized in the cell nucleus upon HCMV infection or UL97 transfection. In addition, pUL97 was detected at the perinuclear compartment, the nuclear membrane, and the cytoplasm. During the HCMV replication cycle, pUL97 is involved in the regulation of viral DNA synthesis taking place in the nucleus, and in processes at the nuclear membrane (like the nucleocytoplasmic capsid export), and possibly in the cytoplasm.

Deletion of the UL97 gene from the viral genome resulted in a drop of HCMV replication by 2 orders of magnitude, demonstrating the necessity of active pUL97 for HCMV. Based on these observations, the search for potent, selective, and nontoxic pUL97 kinase inhibitors easily penetrating into human cells should result in efficient drugs for the treatment of HCMV infection.

Using the baculovirus expression system for insect cells, we obtained the active full-length pUL97 protein, which we used for screening a compound library derived from known protein kinase inhibitors covering 60 different scaffolds. The in vitro kinase activity was examined in the presence of radiolabeled γ-32P-ATP and quantified by measuring phosphorylation of myelin basic protein (MBP), a mutual protein kinase substrate.

Cells stably transfected with the UL97 gene acquire sensitivity to GCV cytotoxicity for reasons described above. Based on this feature, we have established a screening system, which allows the identification of inhibition of pUL97 in intact cells. The principle of this in-cell activity assay is the intracellular inhibition of pUL97 kinase activity by compounds added to the culture, thus preventing the cytotoxic effect of GCV in pUL97-transfected cells. In the present study, we developed this assay further for large-scale screenings of pUL97 inhibitory compounds.

The hits identified in the 2 independent screening systems defined diverse groups of compounds matching the requirements of the respective assay. Interestingly, fractions of each group were overlapping, and these compounds represent promising candidates for further drug development.

MATERIALS AND METHODS

Materials

TC-100 insect cell medium was purchased from Biochrom AG (Berlin, Germany). All other culture media and supplements as well as MBP were from Gibco BRL/Life Technologies (Groningen, the Netherlands), and geneticin (G418) was from Invitrogen (Karlsruhe, Germany). The Large Construct Kit for the isolation of recombinant transfer vector was obtained from Qiagen (Hilden, Germany). Glutathione sepharose was from Amersham Biosciences (Freiburg, Germany). GCV (Cymeven) was from Hoffmann–La Roche (Grenzach, Switzerland), and the staurosporine derivative, nonglycosidic indolocarbazole I (NGIC-I), was from Calbiochem (#481500). The compound library, consisting of kinase inhibitors, was synthesized by Vichem Ltd (Budapest, Hungary) and will be published elsewhere. All other chemicals were from commercial sources and of the highest purity available.

Disposable cell culture material and 96-well U-bottom plates (#650161) were from Greiner (Frickenhausen, Germany). FlashPlates.SMP106 were from PerkinElmer (Brussels, Belgium), and 20-µL tips (#901-252) and 200-µL tips (#903-252) were from Fisher Scientific (Ingolstadt, Germany).

Cell lines and culture conditions

Human embryonic kidney (HEK) 293 cells (American-type culture collection, ATCC CRL-1573) were grown in Dulbecco’s modified minimal essential medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Every 3 to 4 days (i.e., before reaching confluency), cells were subcultured (1:5) in fresh medium after treatment with 5 mg/mL trypsin and 2 mg/mL ethylenediamine tetraacetic acid (EDTA) dissolved in sterile phosphate-buffered saline (PBS).

HEK293 cells were stably transfected with a geneticin-resistance plasmid containing the complete open reading frame of the cytomegalovirus (CMV) gene UL97 (293UL cells). Transfectants were grown in the presence of 0.5 mg/mL geneticin (G418) as the selective agent for the maintenance of the pUL97 plasmid. These cells were used for maximally 10 passages before a new frozen batch was seeded.

Spodoptera frugiperda Sf9 cells (ATCC CRL-1711) and Sf21 cells (DMSZ ACC 119) were grown in TC-100 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine.

Construction of glutathione S-transferase–pUL97 baculovirus

For expression of GST-pUL97 in insect cells, we followed the instructions provided by Invitrogen (Paisley, UK). A full-length UL97 cDNA fragment was obtained by digesting a recombinant UL97-pcDNA3 expression plasmid with EcoRI/XhoI and was cloned into the baculo-transfer vector pFASBAC1. A fragment containing the glutathione S-transferase (GST) cDNA was amplified by PCR using 5 ng of pGEX5x1 vector as template and using a GST-specific primer with a 5’ EcoRI site and an M13 forward primer. The PCR fragment was digested with EcoRI, and the re-
sulting 684-bp GST sequence was cloned upstream of the pUL97 open reading frame into the pFASTBAC1 vector. The correct orientation of the GST gene was controlled by digestion with EcoNI, yielding a 1668-bp and a 5909-bp fragment. A kinase-inactive version of pUL97 was constructed by changing the lysine at amino acid position 355 to a methionine with the help of oligonucleotide-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). Recombinant pFASTBAC1 vector was transformed into MAX Efficiency® DH10BAC™ competent *Escherichia coli* cells (Invitrogen, Paisley, UK) containing both *Autographa californica* nuclear polyhedrosis virus bacmid DNA and a helper plasmid for transposition of the expression cassette from the transfer vector into the baculoviral genome. White bacterial colonies indicating successful transposition were isolated, and baculovirus DNA was controlled for recombination by colony PCR using M13 forward and M13 reverse primer. Recombinant bacmid DNA was isolated using the QIAGEN Large Construct Kit (Qiagen, Hilden, Germany). Isolated DNA (300 ng) was transfected into 1.2 × 10^6 Sf9 cells by lipofection using 6 µL of CellFECTIN reagent (Invitrogen, Paisley, UK). Recombinant baculovirus was harvested 72 h after transfection, and supernatants with higher titer were obtained by successive infection of Sf9 cells. After 4 rounds of virus amplification, sufficient recombinant baculovirus was produced for the expression of pUL97 fusion protein at a large scale. After the fourth round, a virus titer of about 5 × 10^7 pfu/mL was achieved.

**Expression and purification of GST-pUL97 protein**

For pUL97 expression, Sf21 cells were infected with baculovirus carrying a fusion of GST and pUL97 open reading frames (GST-pUL97) at a multiplicity of infection (MOI) of approximately 2. Cells were harvested 69 h after infection, washed once with PBS, and frozen at –80 °C.

Frozen cells from 20 tissue culture flasks (175 cm^2) (Greiner Bio-one, Frickenhausen, Germany) were thawed and resuspended in 20 mL lysis buffer (PBS with 10 mM EDTA, 2 mM dithiothreitol [DTT], 0.5% Triton-X-100, 10% glycerol, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 µg/mL leupeptin, 2 µg/mL aprotinin, 20 µg/mL DNaseI, 10 µg/mL RNaseA) and lysed by sonication (3 × 15 sec and 2 × 30 sec, 70% power; Bandelin Electronic, Berlin, Germany). The homogenate was centrifuged with 30,000g at 4 °C for 30 min. Subsequently, the supernatant was incubated with 1 mL preequilibrated glutathione sepharose 4B (Amersham Biosciences, Freiburg, Germany) for 3 h at 4 °C under rotation. Glutathione sepharose with bound GST-pUL97 fusion protein was collected by centrifugation (3 min, 500g, 4 °C) and washed 3 times with 30 mL wash buffer (20 mM Tris·HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol). GST-pUL97 protein was eluted twice with 1.5 mL elution buffer (90 mM Tris·HCl [pH 8.1], 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol, 30 mM glutathione) for 30 min at 4 °C. The enzyme concentration was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using bovine serum albumin (BSA, 0.1–2.0 mg/mL) as protein standard. In general, a total amount of 200 to 400 µg of purified enzyme was obtained from twenty 175-cm^2 tissue culture flasks. The eluted enzyme was stored in aliquots at –80 °C.

**Cellular assay for pUL97 kinase inhibition**

Details of the principle and initial development of the pUL97 in-cell activity assay have been published in detail.¹⁶ For large-scale screenings, 293-UL cells were seeded in 96-well plates (Greiner 655180) at a density of 1700 cells in 145 µL medium per well, and 24 h later, 50 µL of compound solution (freshly prepared in complete medium containing 0.4% DMSO) and 5 µL of GCV (8 mM) diluted in complete medium were added (final concentration was 200 µM GCV). To evaluate intrinsic drug toxicity, we used another 96-well plate in parallel, which received only the compound (as described above) but no GCV. Each compound was tested in triplicate.

The following controls were implemented in duplicate: wells containing no cells (blank, background control of media), wells with cells plus 0.1% DMSO (positive control, C*), wells with cells plus various amounts of GCV (50, 200, 800 µM), and wells with cells plus the reference inhibitor NGIC-I (3, 10, 30 nM in the presence of 200 µM GCV).

Three days after addition of drugs, cells were fixed by the addition of 40 µL 25% glutaraldehyde and stained with 0.05% methylene blue. Bound dye was solubilized by incubating cells with 3% HCl, and staining was recorded on a Victor II (PerkinElmer Wallac) 96-well reader at 650 nm. Data were exported into an Excel template (Microsoft), and cytotoxicity and GCV protection were calculated.

Experiments were accepted for consideration if the signal-to-background ratio (i.e., growth control vs. reagent blank) exceeded a factor of 10 and if growth inhibition by 200 µM GCV was well above 50%. Data of samples with compounds were used if the standard deviation of triplicates was below 20% of mean value.

Calculation of viability ([drug treated minus reagent blank] normalized by growth control C*) is as follows:

\[
\text{Viability} = \frac{\text{OD with drug} - \text{OD blank}}{\text{OD C}^* - \text{OD blank}}
\]

For nontoxic compounds, values are close to 100, whereas for toxic compounds, values vary between 0 and 50.

Calculation of GCV protection ([drug plus GCV] treated minus GCV treated) normalized by (C* minus GCV treated) is as follows:

\[
\text{GCV Protection} = \frac{\text{OD drug + GCV} - \text{OD GCV}}{\text{OD C}^* - \text{OD GCV}}
\]
For pUL97 inactive, nontoxic compounds, the values are close to 0. For potent pUL97 inhibitors, which are nontoxic, values are close to 100. For toxic compounds, the value is <0.

**GST-pUL97 in vitro kinase assay**

GST-pUL97 kinase activity was monitored by the incorporation of radioactivity from γ-32P-ATP (Amersham Bioscience, UK) (10 μCi/mL) into MBP and by auto-phosphorylation, as published.7,8,16,17 Screening reactions were analyzed by phosphocellulose filter (Whatman P81) binding, washing with 0.75% phosphoric acid, and liquid scintillation counting (Packard Tricarb 2100). A small number of samples were examined by SDS-PAGE and autoradiography of the fixed and dried gel.16

For screening, the pUL97 kinase assay was adapted to 96-well MBP-coated FlashPlates (SMP106; PerkinElmer, Brussels, Belgium) in a 100-μL volume of 20 mM Tris-HCl (pH 7.5), 0.5 mM MnCl2, 1 mM DTT, 1 μM adenosine triphosphate (ATP) containing 0.5 μCi γ-32P-ATP, 0.5 μg GST-pUL97, 1% DMSO, and the indicated drug concentration.

Every plate contained 1 column of control wells: 2 wells with EDTA added prior to enzyme addition (negative control C−, subtracted as blank from all other wells), 2 wells with DMSO control (C0, drug free), and 4 wells with the reference inhibitor in serial 3-fold dilution (NGIC-I; final concentrations were 1.1, 3.3, 10, and 30 nM). On each plate, 88 compounds were examined. All samples were tested in duplicate.

Pipetting was performed on a Multiprobe II/8 8-channel pipetting robot (PerkinElmer/Packard, Frankfurt, Germany) programmed for delivery of 1 μL drug in DMSO, 50 μL ATP dilution (2 μM ATP, 10 μCi/mL), and 50 μL diluted and chilled enzyme.

One hour after addition of enzyme to the first well, the robot started adding 15 μL stop mix (0.1 M NaPO4 [pH 7.2], 0.1 M EDTA) in the same pipetting sequence as for loading the wells, thus enabling the incubation time to be kept at precisely 1 h for all reactions of an experiment (12 × 96 wells for a fully loaded station). Subsequently, liquid was aspirated, and FlashPlates were washed 3 times with 300 μL stop mix per well and counted in a Microbeta reader (PerkinElmer) with the FlashPlate program after a 30-min dark adaptation period.

The counts measured were imported into an Excel template (Microsoft). Averages of counts in the presence of drug were calculated, and negative controls (C−) (reaction in the presence of EDTA) were subtracted from these values and divided by the positive control (C+) (DMSO, but no inhibitor) minus the negative control (C−) resulting in kinase activity as a percentage of the positive control:

\[
\text{Kinase activity} (\%) = 100 \times \frac{(av \ cpm \ with \ drug) - (av \ C^-)}{(av \ C^+) - (av \ C^-)}
\]

For analysis, all data were imported into the databank system ActivityBase (IDBS). Signal-to-background ratios (C+ divided by C−) between 5 and 10 were usually achieved, and standard deviations of duplicates were generally <25%. Enzyme amounts were adjusted to achieve a turnover of 1% to 5% of the ATP. The assay provided reproducible results, with the standard deviation of C+ being <5% within 1 experiment and <15% between different experiments. The Z′ factor was usually >0.7, and experiments with Z′ below 0.5 were not considered.

**HCMV GFP-based antiviral infection assay and plaque reduction assay**

Infection assays were performed with HCMV AD169-GFP on human foreskin fibroblasts (HFFs) at 90% confluence in 12-well plates and used for GFP quantification by automated fluorometry, as described previously.16,17 Seven days after incubation, lysates of the infected cells were prepared and used for automated measurement of the GFP signal in a Victor 1420 Multilabel Counter (PerkinElmer/Wallac). Plaque reduction assays were performed by the use of a 0.3% (w/v) agar overlay on infected cells and staining of viral plaques with 1% crystal violet 8 to 12 days postinfection.16,17 Antiviral drugs were added to the culture medium and agar overlay immediately after virus adsorption.

**RESULTS**

**Inhibitory effect of compounds in pUL97 in vitro kinase assays**

The viral protein kinase pUL97 was expressed in insect cells, purified, and enzymatic activity tested. As a vital control, we purified a kinase-inactive mutant of pUL97 (K355M) in parallel. Although the wild-type pUL97 preparation contained high kinase activity, demonstrated by MBP and auto-phosphorylation, no activity was detected in the UL97 (K355M) mutant fraction (data not shown). This finding indicated that purified pUL97 was devoid of contaminating kinases.

Time course experiments revealed that the enzymatic kinase reaction was proportional to incubation time for more than 1 h (i.e., as long as the total ATP consumption was below 10%; data not shown). Under screening conditions, we incubated for 60 min with enzyme amounts (0.6 nM), allowing consumption of 1% to 5% of total ATP.

A compound library consisting of 4978 protein kinase inhibitors was tested for their inhibitory activity on purified pUL97 kinase. A total of 971 compounds belonging to different chemical scaffolds inhibited the kinase activity by more than 50% at a concentration of 10 μM. It turned out that the class of indolocarbazoles contained quite efficient inhibitors. Therefore, some members of this class were tested at various concentrations, and IC50 values were determined. For example, NGIC-I (Fig. 1), a staurosporine derivative, showed an IC50 value of 12 ± 3 nM, which correlates well with literature data.16,17 Therefore, NGIC-I served as a control for screening accuracy, and the IC50 values for NGIC-I were determined in every screening experiment.
Properties of pUL97-transfected cells

In contrast to nontransfected HEK293 cells, cell lines stably transfected with pUL97 were highly sensitive in a dose-dependent manner to GCV, leading to growth arrest and cytotoxicity (Fig. 2). Addition of the pUL97 kinase inhibitor NGIC-I (Fig. 1) allowed growth of pUL97-transfected cells, even in the presence of GCV (Fig. 3). Thus, NGIC-I was able to penetrate the cellular membrane and to block pUL97 activity within the cell. This example demonstrated the merits of the established assay for testing pUL97 inhibitors in intact cells. Therefore, the system was employed for screening the kinase inhibitor library from the Axxima AG compound archive.

Inhibition of pUL97 kinase activity in the cellular assay

For the selection of compounds qualified for drug development, we tested our library consisting of 4978 compounds at 10 and 1 µM for toxicity and for their potential to inhibit pUL97 in UL97-transfected cells (293-UL cells). More than 80% of all compounds turned out to be nontoxic, and 840 compounds out of the 971 pUL97 inhibitors did not affect the growth of cell culture (Fig. 3).
An important criterion for drug development is cellular efficacy. From the 840 nontoxic pUL97 inhibitors, 93 were able to protect the 293-UL cells from the cytotoxic effect of GCV (Fig. 4A, group d). Many of these compounds showed this protection in a dose-dependent manner, as exemplified for NGIC-I (Fig. 3B). There was a striking correlation ($r = 0.8$) between inhibition of pUL97 in vitro (10 $\mu$M) and protection of 293-UL cells against GCV (Fig. 4C).

In total, the characteristics determined (in vitro kinase inhibition, cytotoxicity, and GCV protection) allowed the classification of all compounds into 7 distinct groups (Fig. 4B; for details, see figure legend). Characteristic representatives for some of these groups are depicted in more detail in Figure 5A-C. Interestingly, a group of 106 compounds protected cells from GCV toxicity but did not inhibit pUL97 activity in the in vitro kinase assays (Fig. 4B, group b).

To discover more compounds like NGIC-I,16,17 which were able to block pUL97 and HCMV, we performed both HCMV replication and plaque reduction assays. The benzimidazole Ax 6438 (Fig. 6A), a potent, nontoxic in vitro pUL97 kinase inhibitor ($IC_{50}$ was 0.1 $\mu$M) protecting against GCV toxicity (Fig. 5), was able to block HCMV replication (Fig. 6B) and reduced plaque formation (Fig. 6C) at a similar magnitude as GCV. Another promising example of this group were quinazolines, which will be described in
In total, we analyzed 26 compounds of the nontoxic, GCV-protective pUL97 inhibitors (Fig. 4B, group d). Seventeen of them, such as Ax 6438, had an IC₅₀ ≤ 10 µM in HCMV replication assays, whereas 9 did not show a significant inhibition of HCMV (data not shown).

FIG. 5. Examples of compounds from pUL97 screenings. Typical representatives from groups defined in Figure 3B are depicted. Data are presented as (A) relative protection from ganciclovir (GCV) cytotoxicity, (B) relative toxicity by the compound itself, and (C) percent inhibition of pUL97 in vitro at the respective concentration (1 and 10 µM). Letters in brackets refer to the groups according to the scheme and definition in Figure 4. For more details, see also the legend of Figure 4.
DISCUSSION

The goal of this study was the identification of inhibitors for the kinase pUL97 from HCMV. Therefore, compounds were screened in an in vitro kinase assay and in a cellular assay for the determination of cytotoxic and pUL97 inhibitory properties.

Nearly 20% of the compounds showed in vitro pUL97 inhibitory activity. This confirms that the collection is focused on protein kinase inhibitors and, furthermore, that the pUL97 ATP binding pocket allows access and binding of a variety of compounds and scaffolds. In this context, it is interesting to mention that the pUL97 kinase contains a bulky methionine residue at the “gatekeeper” position (amino acid 418), which may prevent access to the hind pocket of the ATP binding domain and thus may hinder binding of many more inhibitors.19 However, only knowledge of the structure will ultimately allow the design of rationally highly potent and specific inhibitors for pUL97.

For the cellular analysis, HEK293 cells were stably transfected with the UL97 cDNA, which rendered cells sensitive to cytotoxic effects caused by GCV. These cells increased their susceptibility by about 75-fold, and IC_{50} were 3700 µM and 50 µM GCV for wild-type and UL97-transfected cells, respectively (Fig. 2). This

FIG. 6. (A) Structure of Ax 6438. Ax 6438 is a benzimidazole, which inhibits pUL97 with an IC_{50} of 0.1 µM ± 23 nM in the in vitro kinase assays. (B) Human cytomegalovirus (HCMV) green fluorescent protein (GFP)-based infection assay. For measurements of viral replication by the GFP fluorometry, human foreskin fibroblasts (HFFs) were grown in 12-well plates and infected with 0.25 GFP-forming tissue culture infectious doses of HCMV AD169-GFP. Compound Ax 6438 or reference inhibitor GCV was diluted in culture medium at the concentrations indicated (1.1, 3.3, or 10 µM) and added to the cell cultures after virus adsorption. Infection rates (GFP-positive cells) and the lack of cytotoxicity of the compounds (confluent cell layers) were monitored daily by microscopy. Seven days postinfection, cell layers were harvested, lysed, and subjected to the automated fluorometry of GFP signals. The IC_{50} for Ax 6438 and GCV were 8 and 2 µM, respectively. Each panel refers to a determination in quadruplicate (infection in duplicate, measurement of lysate samples in duplicate). Mock: no addition of virus HCMV AD169-GFP. DMSO: virus added in presence of DMSO solvent (0.1%). (C) HCMV plaque reduction assay. HFFs were infected in 12-well plates with HCMV AD169 and overlaid with the agarose medium containing the indicated concentrations of antiviral compounds (Ax 6438 and GCV). Eight days postinfection, virus plaques were stained and quantified by microscopic counting (infection in duplicate, counting in duplicate). Ax 6438 efficiently blocked formation of HCMV plaques.
in-cell activity system proved to be a valuable tool for the identification of cellular active, nontoxic pUL97 inhibitors.

The transfected HEK293-pUL97 cells did grow slightly slower (by about 20%) than wild-type or mock-transfected cells (data not shown), which may indicate that pUL97 kinase is conferring a growth disadvantage to cells expressing this foreign protein. Furthermore, upon repeated subculturing, the cells tend to lose their capacity to express enzymatically active pUL97 kinase, indicated by a decreasing responsiveness of the culture to the cytotoxic effect of GCV. Therefore, new stocks of frozen cells had to be used after several rounds of subculturing.

We tested the collection of nearly 5000 kinase inhibitor candidates in this in-cell activity assay to determine (1) the intracellular inhibition of pUL97 and (2) the effect of the compounds on cell viability. According to these results, the compounds could be grouped into 3 classes—namely, those that inhibited pUL97 in vitro, those that blocked pUL97 activity in cells, and those that were cytotoxic. These 3 major classes were partly overlapping, thus resulting in 7 subclasses (Fig. 4B, groups a-g). The inhibition of pUL97 in vitro, toxicity, and the protection against GCV cytotoxicity of 5 typical representatives (groups a, b, d, f, and g) are depicted in Figure 5. Ax 6438, a benzimidazole, was cellular active and nontoxic (group d); Ax 3630, a flavonoid, was cellular active but toxic (group g); Ax 11292, a pentose-substituted benzimidazole, was cellular active but inactive in the in vitro kinase assays (group b); Ax 8497, a polycyclic acarone, was in vitro active but cellular inactive (group a); and Ax 3546, an oxindole, was in vitro active and toxic (group f). The indolocarbazole NGIC-I, which was not toxic, blocked pUL97 in vitro, and protected pUL97-transfected cells against GCV (Fig. 4B, group d), showed high HCMV-specific antiviral potential (EC_{50} value of 38.5 nM) by a mechanism directly targeting the viral protein kinase pUL97.

Here we present another example—namely, the benzimidazole Ax 6438, which represents a promising candidate for HCMV drug development (Fig. 6).

About half of the compounds protecting pUL97-transfected cells from GCV demonstrated a good correlation with the inhibition of pUL97 activity in vitro (Fig. 4B,C). However, unexpectedly, many compounds defending cells from GCV toxicity lacked in vitro pUL97 kinase inhibitory activity (group b). The molecular mechanism underlying this phenomenon has to be investigated in more detail. Reasons may be that these compounds disturb the cellular uptake of GCV or inhibit cellular kinases, which phosphorylate GCV monophosphate further to the triphosphate.

On the other hand, a number of the in vitro pUL97 kinase inhibitory compounds did not protect cells from the toxic effect of GCV. An apparent explanation is instability of these compounds under cell culture conditions. In addition, this group of compounds may contain inhibitors unable to penetrate the cell membrane, and elucidating this property was one of the major reasons for us to establish the UL97-transfected cell line. Another reason for failure in the in-cell assay might be that the compounds do not enter the subcellular compartment where pUL97 kinase mainly exerts its activity—namely, the nucleus.

When looking at the entire collection of kinase inhibitor candidates, one may be surprised to observe that 16% (799 out of 4978 compounds) of these compounds showed cytotoxicity at 10 µM. However, one has to consider that we used a nonrandom collection of compounds that had been assembled based on the structures of known, highly active kinase inhibitors. Potent protein kinase inhibitors intrinsically bear the likelihood to hit an essential cellular target and thus be toxic.

However, some compounds were partially protecting against GCV at 1 µM but started to become cytotoxic at 10 µM. Therefore, the data described here emphasize the importance of cellular screenings also at various low, nontoxic concentrations to prevent missing compounds that could be used for further drug development.

In conclusion, we show that a large proportion of compounds identified as in vitro pUL97 kinase inhibitors were also detected in the cellular GCV protection assay and vice versa (Fig. 4). Both cellular and in vitro screenings have their merits, and to run both systems in parallel increases the probability to identify compounds that may enter successful drug development.

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Combining In Vitro and Cellular Screening Assays


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