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Protein kinase inhibitors of the quinazoline class exert anti-cytomegaloviral activity *in vitro* and *in vivo*

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Abstract

Cytomegalovirus infection is associated with severe disease in immunocompromised individuals. Current antiviral therapy faces several limitations. In a search of novel drug candidates, we describe here the anti-cytomegaloviral properties of two compounds of the chemical class of quinazolines, gefitinib (Iressa®) and Ax7396 (RGB-315389). Both compounds showed strong inhibitory effects *in vitro* against human and animal cytomegaloviruses with IC_{50}s in a low micromolar range. Cytotoxicity did not occur at these effective concentrations. The antiviral mode of action was based on the inhibition of protein kinase activity, mainly directed to a viral target kinase (UL97/M97) in addition to cellular target candidates. This was demonstrated by a high sensitivity of the respective protein kinases *in vitro* and by infection experiments with viral mutants carrying genomic alterations in the ORF UL97/M97 modulating viral drug sensitivity. In a guinea pig model, gefitinib showed inhibition of cytomegaloviral loads in blood and lung tissue. Importantly, the rate of mortality of infected animals was reduced by gefitinib treatment. In contrast to the *in vitro* data, Ax7396 showed no significant antiviral activity in a mouse model. Further *in vivo* analyses have to assess the potential use of gefitinib in the treatment of cytomegalovirus disease.

Keywords: Human cytomegalovirus; Protein kinase inhibitors; Antiviral activity; Mode of action; Animal models; Drug development

1. Introduction

Human cytomegalovirus (HCMV) is a member of the family *Herpesviridae* and is associated with severe forms of human diseases (Mocarski et al., 2007). Primary acute infection as well as lifelong persistence can lead to life-threatening clinical manifestations particularly in the immunocompromised host. High risk of HCMV disease is linked to transplant, tumor and AIDS patients as well as to neonates. The clinical application of presently available anti-cytomegaloviral drugs faces severe limitations, such as the induction of adverse side effects and the selection of drug-resistant viruses. Thus, the development of improved drugs and therapy regimens is in the focus of worldwide investigations.

The HCMV-encoded protein kinase pUL97 represents an interesting novel drug target. pUL97 is important for the efficiency of viral replication, as this kinase phosphorylates a number of viral and cellular proteins at early and late stages of viral replication, i.e. pUL44, EF-1δ, p32, lamins and RNAP II (Kawaguchi et al., 2003; Krosky et al., 2003; Marschall et al., 2003, 2005; Baek et al., 2004; Romaker et al., 2006). pUL97-specific inhibitors proved to be powerful anti-cytomegaloviral agents both *in vitro* and *in vivo* (Zimmermann et al., 2000;

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Biron et al., 2002; Marschall et al., 2002; Herget et al., 2004). Maribavir (1263W94) is a compound of the chemical class of benzimidazole L-ribosides possessing pUL97-inhibiting activity and possibly secondary effects on other viral proteins (Biron et al., 2002; Chou et al., 2004, 2007). In clinical phase I and II studies, maribavir showed clear antiviral activity and a very promising pharmacological profile (Koszalka et al., 2002; Lalezari et al., 2002; Wang et al., 2003a). Further clinical experimentation will have to prove the success of this drug candidate.

Another rationale for the investigation of inhibitors of viral protein kinases resulted from the approval of protein kinase inhibitors for cancer therapy (Herbst et al., 2004; Buschbeck, 2006; Herget and Marschall, 2006). It seemed predictive that anti-tumoral drugs and related compounds of the same chemical classes may possess additional antiviral activity. Recently, a strong antiviral activity was proven for quinazoline compounds (Herget et al., 2004). Three related quinazolines, i.e. Ax7396, Ax7376 and Ax7543, were identified as potent inhibitors of the pUL97 kinase activity by blocking pUL97 substrate phosphorylation. Replication of HCMV in primary human fibroblasts was efficiently suppressed with IC_{50} values in the range of the current therapy standard ganciclovir (GCV). Importantly, a strong inhibitory effect of quinazolines was demonstrated for clinical HCMV isolates including those resistant to GCV- and cidofovir (CDV) treatment. Quinazolines inhibited viral early-late protein synthesis but did not have effects at other replicative stages such as viral entry (Herget et al., 2004). Therefore, the mode of action of these quinazoline compounds was primarily confined to the inhibition of the pUL97 protein kinase but secondary effects seemed possible (e.g. an additional targeting of cellular protein kinases). The drug gefitinib (Iressa®) also belongs to this chemical class and is a protein kinase inhibitor approved for use in the therapy of small cell lung cancer (SCLC; Cohen et al., 2003). Gefitinib is a known inhibitor of the cellular epidermal growth factor receptor (EGFR) (Ono and Kuwano, 2006). The EGFR is a member of the erbB subfamily of receptor tyrosine kinase proteins. EGFR activation on the cell surface of cancer cells is believed to promote signaling cascades, cell growth, cell cycle progression, differentiation and other events. Inhibitors of EGFR, such as gefitinib (Iressa®) or erlotinib (Tarceva®) belong to a novel generation of anti-tumoral drugs (Herbst et al., 2004; Brehmer et al., 2005). Interestingly, gefitinib also exerts an anti-HCMV activity *in vitro* as indicated by a previous study, albeit at a lower level of efficacy compared to the related quinazolines Ax7396, Ax7376 and Ax7543 (Herget et al., 2004). The intracellular signaling initiated by the activation of EGFR kinase activity is considered as an important driving force of HCMV infection and gene expression (Fortunato et al., 2000). Conflicting results were published concerning the question whether EGFR may function as an entry receptor for HCMV (Wang et al., 2003b; Isaacson et al., 2007).

In this study, we analyzed the antiviral activity of the quinazolines gefitinib and Ax7396. Both compounds showed marked inhibitory activity in cell culture-based virus replication systems and were subsequently investigated in cytomegalovirus-infected animals. The results are partly promising and partly unexpected so that the potential use in further antiviral drug development is discussed.

2. Materials and methods

2.1. Cell culture and viruses

Primary human foreskin fibroblasts (HFFs), human IMR90 cells, mouse (MEFs), rat (REFs) and guinea pig embryonic fibroblasts (GPEFs) were cultivated in MEM containing 5–7.5% (v/v) fetal calf serum (FCS). HCMVs AD169, AD169-GFP (Marschall et al., 2000), GDGrP53 (Sullivan et al., 1993), GDGrXbaF4 (Sullivan et al., 1992) and 759rD100 (Biron et al., 1986) were propagated in HFFs and virus titers were determined by plaque assay or automated GFP fluorometry, respectively (Marschall et al., 2000). MCMVs strain Smith (wt), MCMV-GFP (Mathys et al., 2003), MCMV-delM97 (Wagner et al., 2000), MCMV-UL97 (Wagner et al., 2000) and GCV-resistant mutant SmG80 (Scott et al., 2005) were propagated in mouse fibroblasts, RCMV strain Maastricht (Kaptein et al., 2006) in rat fibroblasts and GPCMV-GFP (McGregor and Schleiss, 2001) in guinea pig fibroblasts, respectively. 293T cells were cultivated in DMEM containing 10% FCS and used for transfection experiments to produce recombinant proteins.

2.2. Antiviral compounds

Antiviral drugs Ax7396 (RGB-315389) and gefitinib (Iressa®) were freshly synthesized and provided from the stocks of GPC Biotech AG. Reference drugs with known antiviral or antiproliferative activity were used as follows: ganciclovir (GCV, Cymevene; Syntex-Arzneimittel/Roche), cidofovir (CDV, Vistide; Pharmacia & Upjohn S.A., Luxembourg), staurosporine (STP, Calbiochem/Merck) and taxol (Paclitaxel; Calbiochem/Merck). For use in cell culture, stocks were prepared in aqueous solution (GCV and CDV) or in DMSO (STP and taxol) and aliquots were stored at −20 °C. For use in animal experiments, the compounds were dissolved in 3% DMSO/pyrogen-free saline (Ax7396 and GCV) for intraperitoneal (i.p.) administration to mice, or in a 25 mg/ml-resuspension of the lyophilized powder in 0.5% polysorbate 80 (gefitinib; cyclic CDV in PBS) for oral administration to guinea pigs, respectively.

2.3. Cytomegalovirus GFP-based antiviral assay

HFFs or MEFs were cultivated in 12-well plates and used for infection with a recombinant green fluorescent protein (GFP)-expressing cytomegalovirus, i.e. HCMV AD169-GFP or MCMV-GFP, at a multiplicity of GFP-TCID 0.25 (i.e. 25% GFP-forming tissue culture infectious dose). All infections were performed in duplicate; GFP quantifications were performed in quadruplicate. The assay was carried out as described previously (Marschall et al., 2000; Rechter et al., 2006). Briefly, at 7 days post-infection (HCMV) or 5 days post-infection (MCMV), cells were lysed and the lysates from each well were divided into two independent samples and subjected to automated GFP quantifi-

cation in a Victor 1420 Multilabel Counter (Perkin Elmer Wallac GmbH, Freiburg, Germany).

2.4. Plaque reduction assay

Infection assays in cell culture were performed with stocks of cytomegalovirus in 12-well plates at 90% confluence. Following virus adsorption at 37 °C for 90 min, inoculi were removed and 0.3% agarose overlays, optionally supplemented with antiviral compounds, were added to the wells. The staining of viral plaques with 1% crystal violet was performed 5–10 days post-infection. All infections and microscopic countings were performed in duplicate. For the analysis of tissues samples from animal experiments, the excised samples were weighed and homogenized through a sterile filter (BD Falcon Cell Strainer, REF 352350). The resulting homogenates (volumes of 1–1.5 ml) were used as infectious inoculi for plaque titrations.

2.5. Cytotoxicity and cell viability assays

As described previously (Rechter et al., 2006), HFFs were grown in 48-well plates to subconfluent layers and then incubated with antiviral compounds in the culture media (without phenol red) for 5, 7 or 10 days. Medium samples were taken and assayed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) to determine the lactate dehydrogenase (LDH) released from nonviable cells via color substrate conversion. For this, cell debris was removed by centrifugation of the medium samples and 50 μl of each sample was incubated with 50 μl of the substrate mix for 30 min in the dark. The photometric determination was performed via ELISA Reader (OD 490 nm). For a dye exclusion assay, 48-well plates were cultivated with HFFs or MEFs and incubated with antiviral compounds for 5 or 7 days before staining with trypan blue (0.2% trypan blue incubated for 15 min at room temperature followed by fixation with 10% formaldehyde). Quantification was achieved by microscopic counting of trypan blue-positive areas per well. For a cell viability assay, primary IMR90 cells or HFFs were cultivated in 96-well plates (seeding density 3600/well). After 24 h, cells were treated with compounds by addition to the culture media and incubated for 3 days. Then, a precipitation was performed with 10% TCA and cell mass was determined by staining with sulphorhodamine B (SRB) and a measurement of OD at 520 nm. All determinations were performed at least in triplicate.

2.6. Guinea pig CMV infection model

A guinea pig model was used in which immunosuppressed animals were infected with GPCMV at relatively high MOIs (2×10^5 PFU/animal; virus-induced mortality in most of the infected animals). Young male GPCMV-seronegative Hartley guinea pigs (mean weight ∼350 g) were obtained from Elm Hill Laboratories (Chelmsford, MA). They were randomly divided into four groups of eight guinea pigs. The groups were as follows: GPCMV challenge, gefitinib-treated (group 1); mock-infected, gefitinib-treated (group 2); GPCMV challenge, CDV (cyclic cidofovir)-treated (group 3); and GPCMV challenge, placebo-treated (group 4). All animals were immunosuppressed 48 h prior to GPCMV infection with an i.p. injection of 300 mg/kg cyclophosphamide. On the day of virus challenge, groups 1, 3 and 4 were inoculated i.p. with a GFP-expressing GPCMV at an inoculum of 2×10^5 PFU. Commencing on day 1 following viral challenge, animals in groups 1 and 2 were treated, by oral gavage, with gefitinib at a dose of 100 mg/(kg day), until the death of individual animals or for 19 days when the experiment was terminated. Animals in group 4 were treated with an identical volume of the diluent, 0.5% polysorbate 80, by oral gavage. Animals in group 3 were treated with CDV 20 mg/kg, i.p., on days 1, 7 and 15 post-infection. All animals received a second dose of cyclophosphamide (100 mg/kg, i.p.) at day 7 post-challenge. Weights were recorded daily for 19 days. Bleeds were performed on days 5 and 14 post-infection by toe clip 1 h after treatment for quantitative PCR analysis of viral load and for plasma analysis. On day 19 post-infection, all animals who remained alive were sacrificed 1 h after treatment. Heart sticks were performed for quantitative PCR analysis of viral load, along with plasma collection. Lungs were removed at necropsy and dounce-homogenized (10%, w/v) for subsequent DNA extraction and PCR analysis.

2.7. Mouse CMV infection model

A mouse model was used in which immunocompetent animals were infected with MCMV at moderate MOIs (5×10^5 PFU/animal; absence of virus-induced mortality). Six to eight-week-old female Balb-c mice (mean weight ∼18 g) were purchased from Charles River Laboratories (Sulzfeld, Germany) and divided into groups of five animals: MCMV challenge, Ax7396-treated (group 1); mock-infected, Ax7396-treated (group 2); MCMV challenge, GCV-treated (group 3) and MCMV challenge, placebo-treated (group 4). Mice were not immunosuppressed and were directly infected with MCMV i.p. at an inoculum of 5×10^5 PFU. Weights were recorded daily. Antiviral treatment was performed by i.p. injections of Ax7396, GCV or placebo starting at 1 day prior infection (−1), followed by 1, 4 and 7 days post-infection (50 mg/(kg day)). On day 10 post-infection, all animals were sacrificed for the preparation of organs. Salivary gland, spleen, liver and lung samples were homogenized for subsequent DNA extraction and PCR analysis or determination of infectious viral titers by plaque assay.

2.8. Determination of drug plasma concentrations

The determination of plasma concentrations of gefitinib in guinea pig plasma samples was performed by standard liquid chromatography/mass spectrometry (LC/MS–MS; Pharmacelsus®, Contract Research Organisation, Saarbrücken, Germany). In brief, samples were separated on a Gemini C6-Phenyl analytical column by gradient elution with ammonium acetate/formic acid and acetonitrile/formic acid. Mass spectrometry was performed on a TSQ Quantum Discovery Max triple quadrupole mass spectrometer equipped with an electro-spray interface (Thermo Electron, Waltham, MA).

2.9. Quantitative PCR analysis

Viral load was monitored in blood and tissue samples using quantitative real-time PCR (qPCR). Total DNA was extracted using the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche; see Fig. 6) or DNeasy Tissue Kit (QIAGEN, Hilden, Germany; see Fig. 7C). For the GPCMV/guinea pig model, qPCR was performed according to the Roche Lightcycler® Probe Design 2.0 program. The following primers, based on the GPCMV gB gene sequence, were employed: forward primer, 5′-CTTCGTGGTTGAACGGG-3′; reverse primer, 5′-GTAGTCGAAAGGACGTTGC-3′; probe 1, 5′-TGGTGACCTTCGTTACCAATCCGTTTGGA-fluorescein; probe 2, 5′-LC red 640-CTTCGTGGTGTTCCTGTTCTGCGT-phosphate. The PCR reaction mixtures were prepared using the Lightcycler® Fast Start Master hybridization probes (Roche) supplemented with additional 2.5 mM $MgCl_2$, 0.5 μM primers and 0.2 μM probes. Each capillary was filled with 15 μl of reaction mix and 5 μl of sample DNA or control DNA. PCR was performed on the Lightcycler® instrument using the following parameters: an initial step at 95 °C for 10 min, then 45 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 15 s and elongation at 72 °C for 15 s. Data was collected by 'single' acquisition during the annealing step. A melting curve analysis was also performed and data acquired in the 'continuous' mode during an increase in temperature from 45 to 85 °C. The sensitivity of the assay was consistently between 5 and 10 copies/reaction. For the MCMV/mouse model, qPCR was performed according to the Applied Biosystems TaqMan® program. Primers were based on the MCMV IE1 gene sequence as follows: forward primer, 5′-TGCCATACTGCCAGCTGAGA-3′; reverse primer, 5′-GGCTTCATGATCCACCCTGTT-3′; probe 5′-CTGGCATCCAGGAAAGGCTTGGTG labeled with 6-carboxyfluorescein reporter dye (FAM) and 6-carboxytetramethylrhodamine quencher dye (TAMRA). The reactions were performed in a 20-μl volume containing either 5 μl of either the DNA sample (1 μg) or a standard DNA solution. Additional components of the reaction mixture were TaqMan® PCR Mastermix (Applied Biosystems), 0.3 μM of each primer and 0.1 μM of the probe. For thermal cycling, conditions consisted of two initial steps of 2 min at 50 °C and 10 min at 95 °C, followed by 40 amplification cycles (15 s at 95 °C, 1 min 60 °C). PCR was performed on the ABI Prism 7700 sequence detector (Applied Biosystems). DNA extracts were analyzed in duplicate and the mean was calculated for all samples from one group of animals.

2.10. Western blot analysis

Fibroblasts were grown in 6-well plates overnight to subconfluency and infected with stocks of cytomegaloviruses at a MOI of 1. 48 h post-infection, cells were lysed in 50 μl RIPA buffer, incubated on ice for 10 min and centrifuged to remove cell debris. Thereafter, 50 μl PAGE sample buffer was added to the lysates. After heating at 95 °C for 10 min, lysates were subjected to standard SDS-PAGE and Western blot detection procedures. Monoclonal and polyclonal antibodies were used for protein detection as follows: MAb-CROMA 101 (MCMV IE1-pp89, S. Jonjic, Univ. Rijeka, Croatia), rabbit polyclonal anti-UL97 (D. Michel, Univ. Ulm), PepAS1459 (peptide antibody recognizing pM97), MAb-GFP (clones 7.1/13.1, Roche) and MAb-β-actin Ac-15 (Sigma).

2.11. In vitro kinase assay

The kinase activity of pUL97 was determined *in vitro* as described previously (Marschall et al., 2001, 2002; Herget et al., 2004; Mett et al., 2005). In brief, after transfection and recombinant expression of pUL97-FLAG in 293T cells, the kinase was immunoprecipitated by the use of MAb-FLAG (M2; Sigma) and incubated with the mutual kinase substrates histone 2B (H2B) or myelin basic protein (MBP) in the presence of [γ-^{32}P]ATP. Radiolabeled proteins were detected by SDS-PAGE and autoradiography. For kinase selectivity panels, enzymes were purchased from Invitrogen (Carlsbad, CA), Upstate (Dundee, U.K.) or ProQinase (Freiburg, Germany). Kinase assays were considered as acceptable when the Z prime value was >0.5. All kinase reactions were performed in the linear range (concerning reaction time and enzyme concentration) and at an ATP concentration according to the K_m of the respective enzyme for comparison of the inhibitory effects of the compounds as described previously (Mett et al., 2005). Each kinase assay was validated with a positive control (i.e. staurosporine, flavopiridol, AG1478, Gleevec/imatinib, VX-680, wortmannin or Y-27632). In addition to measurements by *in vitro* kinase assays, substrate phosphorylation was determined by immobilized metal assay of phosphorylation (IMAP, Molecular Devices; Sportsman et al., 2004). IMAP technology is based on the covalent-coordinate, high-affinity interaction of trivalent metal-containing nanoparticles with phospho-groups (linked to serines, threonines or tyrosines). In brief, fluorescently labeled peptides were phosphorylated in a microwell-format kinase reaction. Addition of the IMAP Binding System induced a specific binding of the phosphorylated substrates which were detected by fluorescence polarization (FP) or time-resolved fluorescence resonance energy transfer (TR-FRET).

3. Results and discussion

3.1. Anti-cytomegaloviral activity of pUL97-inhibiting substances

A library of small molecules designed for kinase inhibition was screened for *in vitro* activity against the HCMV protein kinase pUL97. Hit compounds were identified belonging to several chemical classes. Hereby, the quinazolines gefitinib (Iressa®) and Ax7396 (RGB-315389) showed IC_{50} values for the inhibition of pUL97 kinase activity of 0.40 and 0.35 μM, respectively (Fig. 1). These *in vitro* data could not directly predict a strong antiviral activity, particularly when seen in comparison with the phase III drug candidate maribavir which shows a clearly lower IC_{50} in terms of inhibition of pUL97 kinase activity (app. 100-fold; Biron et al., 2002). However, IC_{50} values *in vitro* do not reliably reflect the sensitivity of

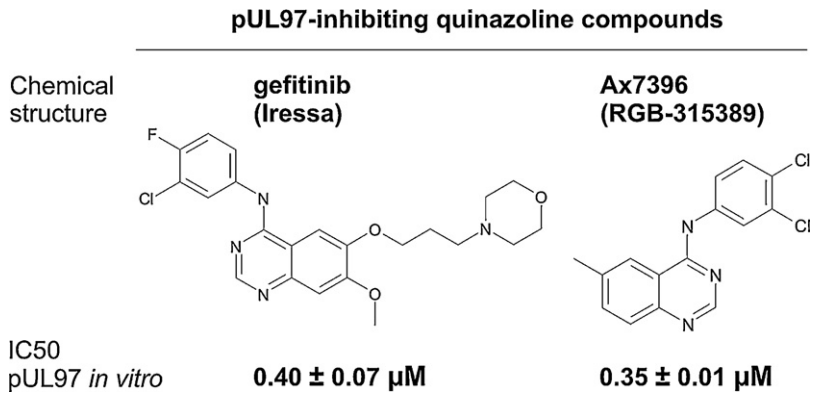

Fig. 1. Chemical structures of pUL97-inhibiting quinazoline compounds and IC_{50} values determined by *in vitro* kinase assays. Distinct quinazoline compounds were selected on a basis to inhibit the viral protein kinase pUL97 *in vitro*. The analysis of inhibitory activity in the *in vitro* kinase was performed as described (Mett et al., 2005) to determine mean inhibitory concentrations (IC_{50}).

a kinase towards inhibitors in cellular systems as being additionally influenced by physiological interactions with substrates and other kinases. Therefore, the selectivity of these compounds was subsequently analyzed in a larger scale of *in vitro* kinase assays and non-radioactive IMAP assays to determine the inhibitory potential of the compounds towards panels of cellular protein kinases. Positive scores were counted as inhibition of 80% or more (Table 1). Interestingly, the two quinazoline-type compounds not only possessed high inhibitory activity against HCMV pUL97, but also against a small number of cellular kinases which may be taken into account as possible secondary targets when assessing anti-HCMV effects. Gefinitib

Table 1
Selectivity panel for two quinazoline-type protein kinase inhibitors

[a] Assay no.	[b] Protein kinase	Gefitinib (Iressa)	Ax7396 (RGB-315389)	Assay no.	Protein kinase	Gefitinib (Iressa)	Ax7396 (RGB-315389)
1	Abl	11	28	25	Lyn	59	18
2	Akt	16	19	26	MAPK3	<1	2
3	Aurka	11	9	27	MAPK8	6	<1
4	Cdk1	39	23	28	MAPK9	6	--
5	Cdk2	4	--	29	MAPK13	<1	--
6	Cdk3	36	--	30	MAPK14	51	--
7	Cdk4	24	--	31	MAP2K1	8	--
8	Cdk5	33	--	32	Met	44	3
9	Cdk6	41	--	33	Pak1	37	5
10	Cdk7	20	--	34	PDGFRA	<1	--
11	Cdk9	97	--	35	PDGFRB	48	14
12	Chek1	18	24	36	Pim-1	30	11
13	Csnk1a1	36	--	37	PKCalpha	<1	8
14	Csnk2b	<1	--	38	Plk-1	7	--
15	EGFR	100	98	39	pUL97	82	94
16	FGFR3	0	<1	40	Raf-1	16	<1
17	Flt3	<1	<1	41	RET	29	<1
18	Fyn	37	14	42	ROCK2	13	5
19	GSK-3beta	7	8	43	SGK	<1	--
20	IGF-1R	<1	83	44	Src	49	52
21	IKKbeta	15	5	45	SRPK1	16	16
22	IR	<1	41	46	TEK	26	<1
23	Kit	14	8	47	YES1	80	--
24	Lck	82	20	48	ZAP70	<1	--

[a]Inhibition of protein kinase activity was determined as described previously, either by [^{33}P-γ]ATP-based *in vitro* kinase assay (Herget et al., 2004; Mett et al., 2005) or non-radioactive IMAP assay (Sportsman et al., 2004). Data are presented as percentage of inhibition at 10 µM of the compounds (% no inhibitor); assays were performed at least in triplicate or reproduced by independent confirmation settings. –, not determined; shaded fields, inhibition ≥80% (cut-off).

[b]*Abbreviations*: Abl, tyrosine kinase c-Abl; Akt, serine/threonine kinase, also known as protein kinase B; Aurka, Aurora serine/threonine protein kinase; Cdk, cyclin-dependent kinase; Chek1, checkpoint kinase 1; Csnk1a1 and Csnk2b, casein kinases 1 alpha 1 and 2 beta; FGFR3, fibroblast growth factor receptor 3; Flt3, Fms-like tyrosine kinase 3; Fyn, Lck, Lyn, Src and Yes, c-Src-family tyrosine kinases; GSK-3beta, glycogen synthase kinase 3 beta; IGF-1R, type 1 insulin-like growth factor receptor; IKKbeta, IkappaB kinase beta; IR, insulin receptor; Kit, tyrosine kinase c-Kit; MAPK3, extracellular signal-regulated kinase ERK1; MAPK8 and MAPK9, c-Jun N-terminal kinases JNK1 and JNK2; MAPK13 and MAPK14, mitogen-activated kinases p38 delta and alpha; MAP2K1, mitogen-activated protein kinase kinase isoform 1, also known as MEK1; Met, receptor tyrosine kinase c-Met; Pak1, Rac-p21-activated kinase 1; PDGFRA and PDGFRB, platelet-derived growth factor receptors alpha and beta; Pim-1, proviral insertion site serine/threonine kinase 1; PKCalpha, protein kinase C alpha; Plk-1, polo-like kinase 1; pUL97, cytomegaloviral serine/threonine kinase; Raf-1, Ras-binding kinase 1; RET, receptor tyrosine kinase 'REarranged during Transfection'; ROCK2, Rho serine/threonine kinase 2; SGK, glucocorticoid-regulated protein kinase; SRPK1, serine-arginine-specific protein kinase; TEK, receptor tyrosine kinase TEK/Tie-2; YES1, nonreceptor tyrosine kinase c-Yes 1; ZAP70, zeta-chain associated tyrosine protein kinase.

Table 2
Anti-cytomegaloviral activity of pUL97 kinase inhibitors in infected cells

Inhibitor	IC50 (μM)[a]			
	HCMV	GPCMV	MCMV	RCMV
Gefitinib (Iressa)	12.0 ± 5.4	3.3 ± 0.6	3.8 ± 2.0	2.4 ± 1.8
Ax7396 (RGB-315389)	6.1 ± 0.3	6.3 ± 3.2	1.6 ± 1.2	6.8 ± 1.2

[a] IC_{50}s are expressed as means ± S.D. of at least two replicate assays and two independent sample evaluations each (i.e. GFP measurements or plaque countings of infected human, rat, mouse or guinea pig fibroblasts in cell culture, respectively). Selectivity of the compounds for the analyzed viruses is indicated by grey shading (cut-off ≤6 μM).

was inhibitory for HCMV pUL97, cellular Cdk9, EGFR, Lck and YES1; A7396 was inhibitory for HCMV pUL97, cellular EGFR and IGF-1R. Particularly remarkable was the finding of Cdk9, since in the field of human immunodeficiency virus, the development of novel pharmacological inhibitors of Cdk9 as putative anti-HIV-1 drugs is the focus of current interest (e.g. candidate drug Indirubin-3′-monoximer; Heredia et al., 2005).

When analyzed for antiviral activity in replication assays with human and animal cytomegaloviruses, promising inhibitory profiles were obtained for gefitinib and Ax7396. Both compounds showed IC_{50} values in a low micromolar range against HCMV and all animal cytomegaloviruses tested, ranging between 1.6 and 12.0 μM (Table 2; Fig. 2A). MCMV replication appeared to be particularly sensitive to inhibition by Ax7396 (Table 2, IC_{50} 1.6 μM). A 5-fold inhibition was noted for HCMV-GFP by both compounds at 30 μM (at 10 μM, app. 4- and 2-fold, respectively; Fig. 2A) and a 10-fold inhibition for MCMV-GFP at 10 μM (Fig. 2C). Next, three HCMV mutants with a drug-resistant phenotype were analyzed. HCMV GDGrP53 carries mutation UL54(A987G) and shows a low-level resistance against GCV and CDV (Fig. 2B left); GDGrXbaF4 carries a short deletion in UL97(Δ590-593) and shows high-level resistance solely against GCV (Fig. 2B middle); 759rD100 is a double-mutant [UL54(A987G)/UL97(Δ590-593)] showing resistance against GCV and CDV (Fig. 2B right; Biron et al., 1986; Sullivan et al., 1992, 1993). Interestingly, these three HCMV mutants showed sensitivity towards both quinazoline compounds. Sensitivity towards Ax7396 was generally high (complete inhibition of all virus mutants at 10 μM; Fig. 2B), while sensitivity towards gefitinib was only intermediate at 10 μM (comparable to the HCMV reference strain shown in Fig. 2A). The higher sensitivity towards Ax7396 might be explained by the combined role of pUL97 and secondary cellular target kinases which might be inhibited in HCMV-infected cells more efficiently by Ax7396 than by gefitinib (e.g. IGF-1R, EGFR or others; see Table 1). Thus, known GCV- or CDV resistance-conferring mutations in UL97 or UL54 do not produce resistance against the quinazolines. This might indicate distinct mechanisms of inhibition. It should be mentioned that GCV does not inhibit pUL97 kinase activity (but GCV is converted by pUL97 to the active GCV monophosphate metabolite), while quinazolines are inhibitors of kinases, such as pUL97 or EGFR, by interfering with the function of their kinase domains (Ono and Kuwano, 2006). Consistent with the findings for HCMV, a GCV-resistant MCMV mutant (carrying a point mutation P497L in the viral kinase gene M97) was also fully sensitive to these compounds (Fig. 2D). Furthermore, this viral mutant showed sensitivity to other pUL97 inhibitors belonging to different chemical classes (data not shown). Thus, as for HCMV, for MCMV, cross-resistance between GCV and quinazolines or other pUL97 inhibitors was not observed.

3.2. Antiviral activity is distinct from cytotoxic effects

It has been demonstrated for several examples that inhibition of protein kinase activities can be associated with an induction of cytotoxicity and a loss of cell viability. This is generally dependent on the drug concentration and the targeted type of protein kinase(s) (Schang et al., 2006; Castoldi et al., 2007). To distinguish between cytotoxic and antiviral activity in this study, three methods were applied to determine cytotoxicity or cell viability, respectively, using three different primary cell types (i.e. HFF, IMR90 and MEF; Fig. 3). In a standard LDH assay, gefitinib and Ax7396 did not induce cytotoxicity of primary human HFFs at concentrations up to at least 10 or 30 μM, respectively. Various incubation periods were analyzed, i.e. 5 days (Fig. 3A), 7 days (Fig. 3B) and 10 days (data not shown), producing very similar results. In parallel, the setting with HFFs shown in Fig. 3B was used for a staining in the trypan blue exclusion assay and the resulting data confirmed the abovementioned degree of cytotoxicity for both compounds. In another trypan blue exclusion staining with primary murine MEFs, the two compounds did not induce notable cytotoxic effects at concentrations below 90 μM (Fig. 3C). This suggests that the level of cytotoxicity is cell type-specific and that murine fibroblasts are less sensitive to high-concentration cytotoxicity induced by gefitinib and Ax7396 than human fibroblasts. Finally, a cell viability assays with primary human IMR90 cells showed no significant signals in the range between 0.1 μM and 10 μM and confirmed the limited degree of cytotoxicity of these compounds at the low micromolar range (Fig. 3D–E).

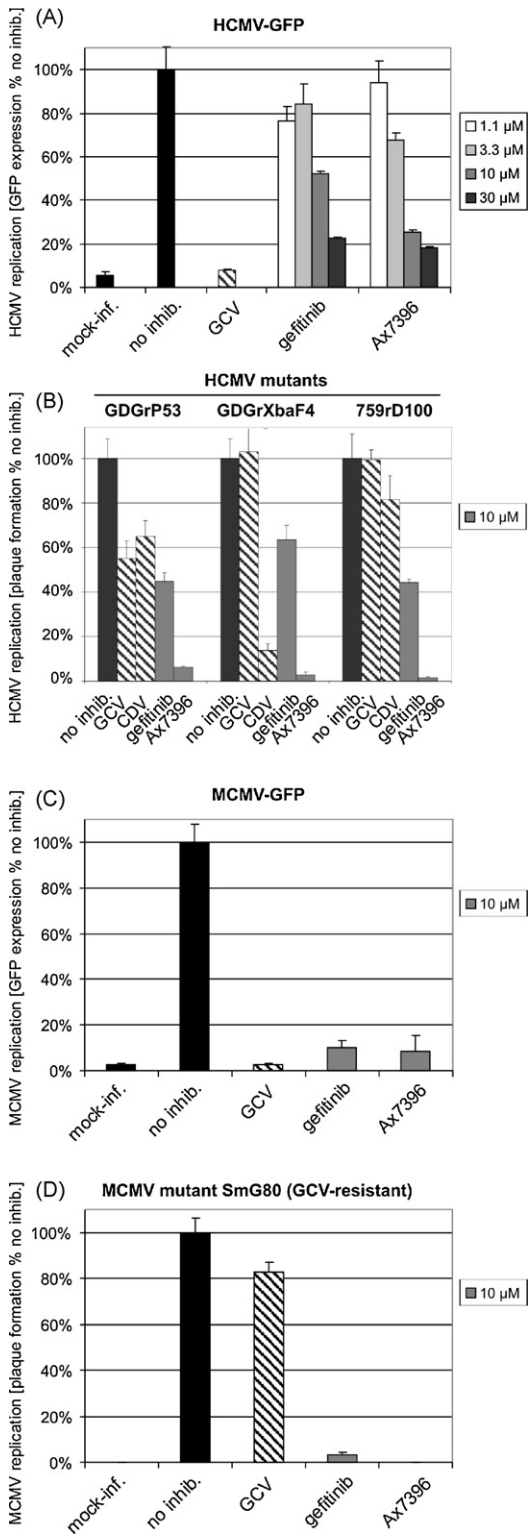

Fig. 2. Comparative analysis of the antiviral potential of gefitinib and Ax7396 towards the replication of three cytomegaloviruses. (A) HFFs were cultivated in 12-well plates and used for infection with HCMV AD169-GFP. Uninfected (mock-infected) or infected cells were incubated in the absence (no inhibition) or presence of inhibitors (concentrations as indicated; GCV 20 μM). Seven days post-infection, cells were harvested and used for a quantitative determination of GFP-signals. A mean of four values is presented in each column. (B) HFFs were cultivated in 12-well plates and used for infection with HCMV mutants (GDGrP53, GDGrXbaF4 and 759rD100). Cells were incubated in the absence or presence of inhibitors as indicated. Quantification of inhibition was performed at 10 days post-infection by plaque staining (mean of four values). (C and D) MEFs were cultivated in 12-well plates and used for infection with MCMV-GFP (strain Smith) or MCMV mutant SmG80 (GCV-resistant), respectively. Inhibitors were applied as follows: GCV 20 μM, gefitinib 10 μM and Ax7396 10 μM. Quantification of inhibition was performed 5 days post-infection by GFP fluorometry or plaque staining, respectively (mean of four values).

For gefitinib, the 50% cytotoxic concentration (CC_{50}) was 26.3 μM for HFFs at 7 days and 54.9 μM for MEFs at 5 days, respectively. This refers to an antiviral index (CC_{50}/IC_{50}) of 2.2 for HFF/HCMV and 14.4 for MEF/MCMV, indicating that the human system shows lower selectivity than the murine system. In the case of Ax7396, the antiviral index was higher in both systems, i.e. 15.2 for HFF/HCMV and 56.3 for MEF/MCMV (CC_{50}s of 92.4 μM and 90.1 μM, respectively). Our cytotoxicity data combined for gefitinib are consistent with published data. It was demonstrated by several studies that for normal cells the gefitinib-induced cytotoxicity is low but for stimulated tumor cells is very strong. As an example, the inhibition of *in vitro* proliferation of stimulated target tumor cells is very efficient (concentration for 50% inhibition determined as 0.054 μM for EGF-stimulated KB cells), while the inhibitory effect on control cells is much less pronounced (50% inhibition at 8.8 μM for nonstimulated KB cells; Wakeling et al., 2002).

3.3. Importance of the cytomegaloviral kinase pUL97/pM97 for the antiviral mode of action of quinazoline compounds

Deletion mutants of MCMV, described by Wagner et al. (2000) were used for experiments analyzing the inhibitory mode of action. The integrity of viral stocks was confirmed by Western blot analysis of samples from infected cells. The expression pattern of viral and recombinant proteins was found to be correct (Fig. 4A). In virus replication assays, a clear concentration-dependent inhibition of MCMV-wt (wild-type) by Ax7396 was measured (Fig. 4B; IC_{50} 1.6 ± 1.2 μM). As an important finding, this sensitivity to the drug was drastically lower in case of a M97-deleted mutant compared to wild-type (Fig. 4C). This mutant, MCMV-delM97, replicates to lower titers and shows a partial replication defect with respect to its parental wild-type (Wagner et al., 2000). However, the low residual replication of this mutant was not subject to inhibition by 1.1 μM or 3.3 μM of Ax7396. Only the highest concentration of 10 μM produced an intermediate level of reduction, possibly due to additional inhibitory effects directed towards cellular protein kinases (e.g. EGFR or other kinases important for MCMV replication; see Table 1). This conclusion was confirmed by the finding that a virus with a genomic replacement of the murine CMV M97 by the human CMV UL97 region regained drug sensitivity (Fig. 4D). This pointed to a targeting of the viral protein kinase (pUL97 or pM97) by Ax7396. In addition, similar experiments were performed with gefitinib, and, although the differences of inhibition between the three MCMVs were less striking, the results suggested a similar conclusion (data not shown).

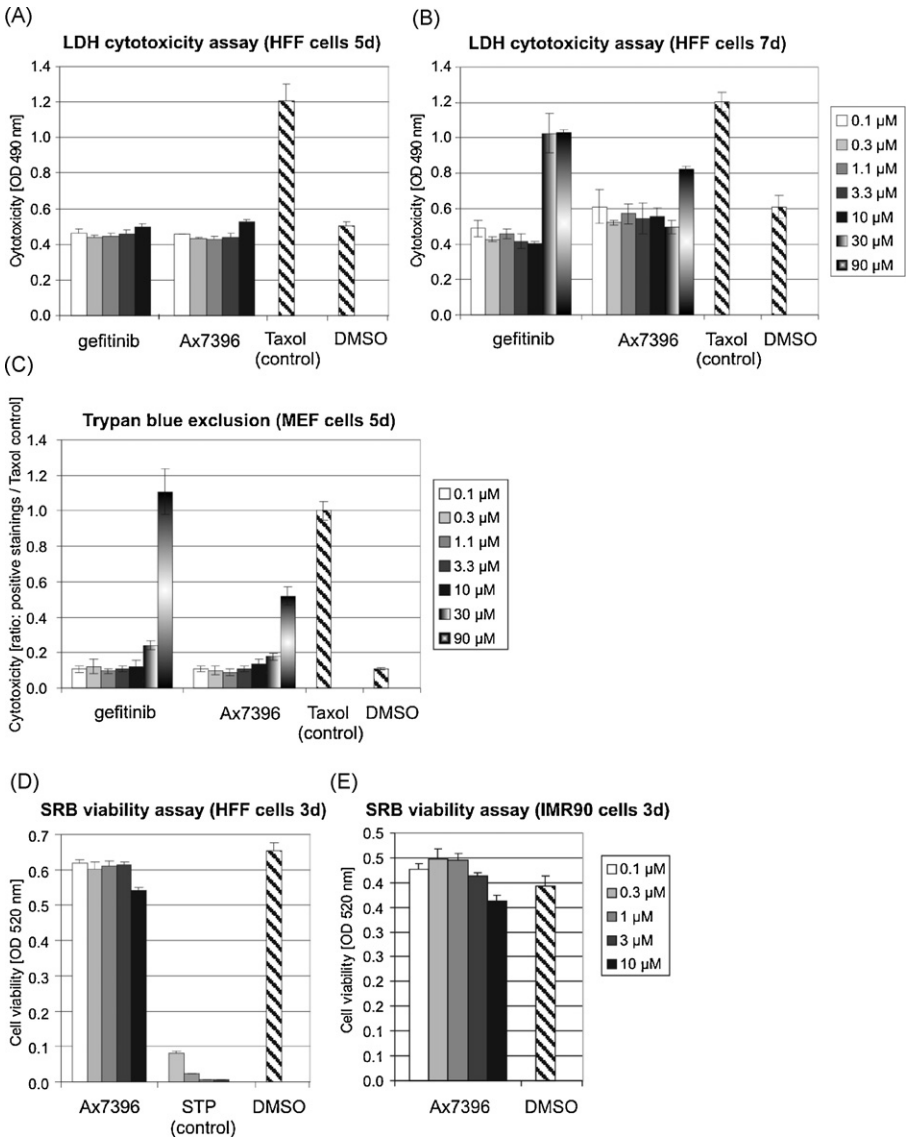

Fig. 3. Determination of cytotoxicity (A, B and C) and cell viability (D and E). Primary cells (HFF, MEF and IMR90) were analyzed in commercially available test systems for the determination of direct cytotoxicity or impairment of cell viability and proliferation. To this end, cells were cultivated in 48-well plates and incubated with the indicated substances in the culture media. After 5 days (A and C), 7 days (B) or 3 days (D and E), samples were taken and assayed according to standard procedures as described. A mean of at least four values is presented in each column. DMSO, solvent control; Taxol (1 μM) and STP (concentrations as indicated), cytotoxicity-inducing controls.

3.4. Gefitinib suppresses the GPCMV-induced mortality and viral loads of infected guinea pigs

As concluded from the *in vitro* analysis, both quinazolines were considered as efficient antiviral compounds (Table 2) and thus, they were further investigated in one of two *in vivo* models. These models were based on distinct experimental parameters which were well-characterized for the individual infection situations in these animal species. In the first model (GPCMV/guinea pig, applied for gefitinib), immunosuppressed animals were used for infection with a relatively high viral dose which was close to the lethal dose under these conditions. In the second model (MCMV/mouse, applied for Ax7396), immunocompetent animals were used for infection with a moderate viral dose, i.e. in the absence of virus-induced mortality. Using two different animal models enabled, on the one hand, to address the specific optimal efficacies of the compounds towards different cytomegaloviruses (see Table 2) and, on the other hand, to compare two related compounds between two different systems. In the guinea pig model, compounds were administered in a continuous regimen; in the mouse model, compounds were only given four times in a discontinuous regimen. Both regimens were adjusted to the point that the control drugs, i.e. CDV or GCV, respectively, showed significant inhibition.

The plasma concentrations of gefitinib in GPCMV-infected animals were determined with serial plasma samples taken over the period of the experiment. The analysis demonstrated that concentrations around the IC_{50} measured for gefitinib in GPCMV-infected cultured cells ($3.3 \pm 0.6\,\mu M$) could also be reached *in vivo* (ranging from 2.50 μM to 4.27 μM; Table 3).

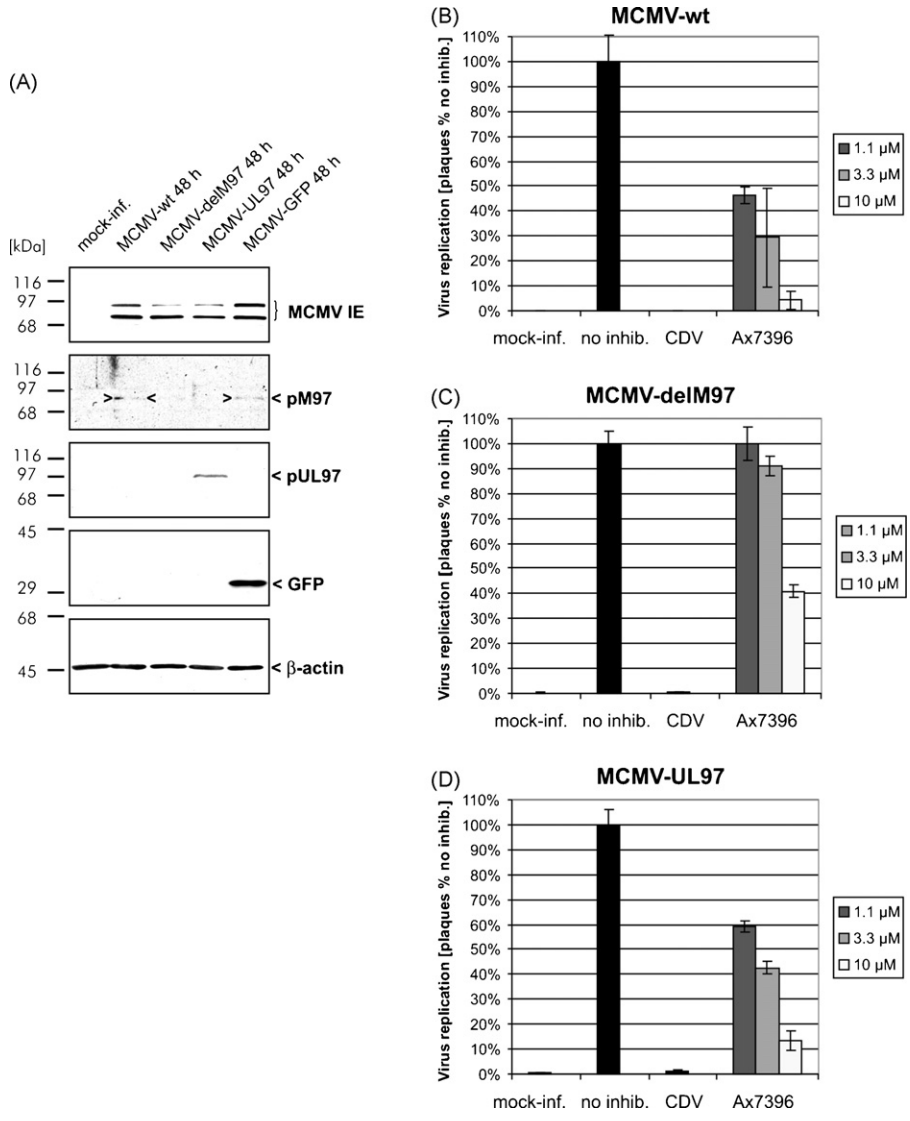

Fig. 4. Sensitivity of viral deletion mutants towards Ax7396. Stocks of MCMV (wild-type and mutants, as described in the text) were used for the infection of murine fibroblasts (MEFs). (A) Total cell lysates were separated by SDS-PAGE and analyzed by Western blot assay using antibodies MAb-IE CROMA 101 (MCMV), anti-UL97 (HCMV), PepAS1459 (pM97), MAb-GFP or MAb-β-actin, respectively, for protein detection. (B–D) For the measurement of antiviral drug-sensitivity of these viruses, MEFs were cultivated in 12-well plates and used for virus infection at low MOI (app. 0.01). Cultivation of infected cells was performed in the presence or absence of the antiviral drugs as indicated. Five days post-infection, plaque formation was quantified. A mean of four values is presented in each column. Uninfected (mock-infected), infected cells without inhibitor (no inhibition), infected cells with cidofovir (CDV, 20 μM).

Published data on human pharmacokinetic levels indicate that gefitinib is extensively distributed in the body with a mean plasma half-life of 39.7 h. Maximum plasma concentrations are typically observed at 5 h after a 250 mg oral dose. A standard Cmax of 79.9 ng/ml (Bergman et al., 2007) equivalent to 0.18 μM by daily application could mean a serious limitation in a potential use as anti-cytomegaloviral drug. This limitation might be addressed by alternative regimens of dosage and routes of application (i.e. intravenous injection).

Here, the effect of gefitinib on animal weights was monitored throughout the course of the experiment and indicated a loss of weight in the GPCMV-infected, placebo group (no inhibition). This effect was slightly reduced in the gefitinib group. An increase of weight was noted in the cidofovir group (Fig. 5A). More importantly, the effect of gefitinib on mortality due to disseminated GPCMV in immunocompromised guinea pigs was analyzed (Fig. 5B). After the 19-day treatment, the total guinea pig mortality in the gefitinib group was 37.5% (3/8), compared to the placebo group (no inhibition), which was 87.5% (7/8). The reduction in mortality was significant in the gefitinib group compared to the placebo control ($P < 0.05$, Chi-squared analy-

Table 3
Plasma concentrations of gefitinib [μM][a] in GPCMV-infected guinea pigs

7 days p.i.	4.27 ± 1.83
14 days p.i.	2.81 ± 0.53
19 days p.i.	2.50 ± 0.97

[a] Gefitinib concentrations were determined from plasma samples by LC/MS–MS; mean values were calculated from $n = 8$ (7 days), $n = 5$ (14 days) and $n = 5$ (19 days).

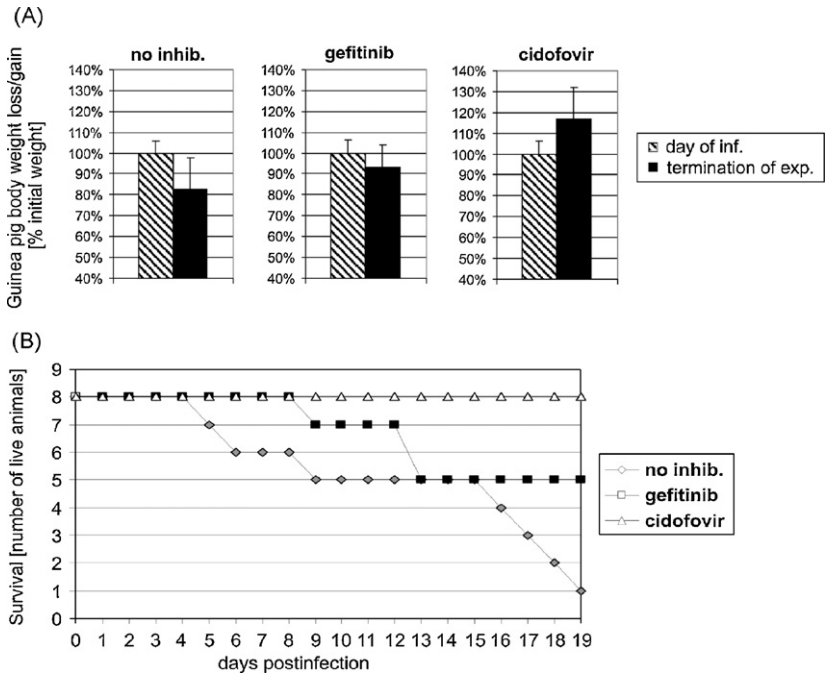

Fig. 5. Guinea pig model: monitoring of body weight and survival of infected animals. Animals were infected with GPCMV and treated with antiviral drugs according to the procedures described in the text. (A) The gain or loss of body weight was recorded daily and a comparison of data from the start and the termination of the experiment is given for the different animal groups. (B) Survival rates are presented for each group of animals along the duration of the experiment (19 days post-infection).

sis). The cidofovir group showed no mortality (0%, 0/8). Thus, gefitinib and cidofovir significantly reduce virus-induced mortality.

The analysis by qPCR showed that gefitinib did not prevent DNAemia, but its use was associated with a reduction in the magnitude of DNAemia (Fig. 6). Viral genomic loads were compared between blood (Fig. 6A) and lung tissue samples (Fig. 6B). Both blood and lung tissue samples showed a reduction of viral load upon gefitinib treatment but the effect was lower compared to cidofovir (note, that cidofovir treatment reduced the genome copy number in blood samples below the limit of detection). No gefitinib-specific differences were found in the magnitude of viral load among positive animals for liver, spleen or kidney (data not shown). Thus, there are no signs for a general block in viral replication/dissemination under gefitinib treatment; however, the reduction of viral loads in the blood and lung compared to the placebo group is in accordance with an increased survival rate.

3.5. Ax7396 appears to be inefficient in antiviral treatment of MCMV-infected mice

Ax7396 was analyzed in a MCMV/mouse model since the IC_{50} determined *in vitro* appeared optimal for MCMV. In this model with normal Balb-c mice, the magnitude of MCMV replication was limited (compared to animals under experimental immunosuppression), but was considered to reflect natural infection in several aspects. Hereby, changes in body weight were neither induced by virus infection nor drug treatment (Fig. 7A). Animals were sacrificed 10 days post-infection for the analyses of tissue samples. In a plaque assay, infectious viral titers were specifically detected in the salivary gland (while other organs

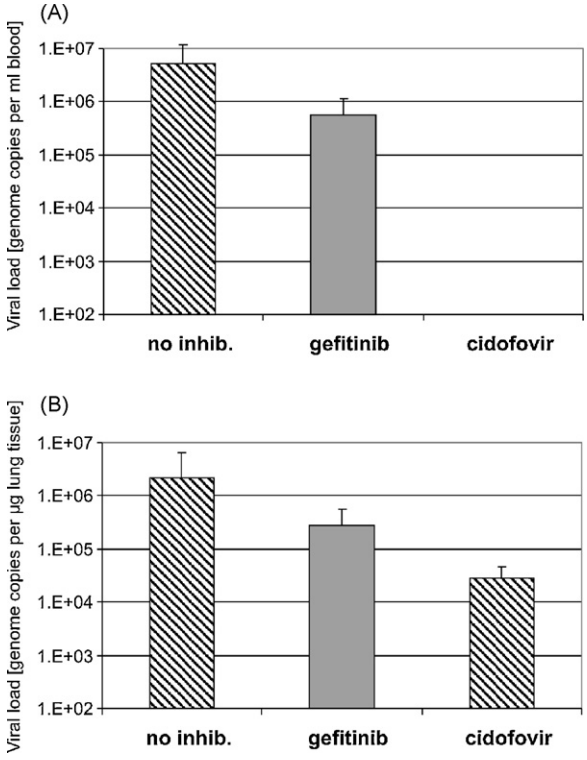

Fig. 6. Guinea pig model: analysis of viral load by qPCR. A quantitative PCR was performed for blood samples (A) and lung tissue samples (B) at the termination of experiment at day 19 post-infection or death of the animal. All qPCR reactions were performed in duplicate and the sensitivity of the assay was consistently between 5 and 10 copies/reaction.

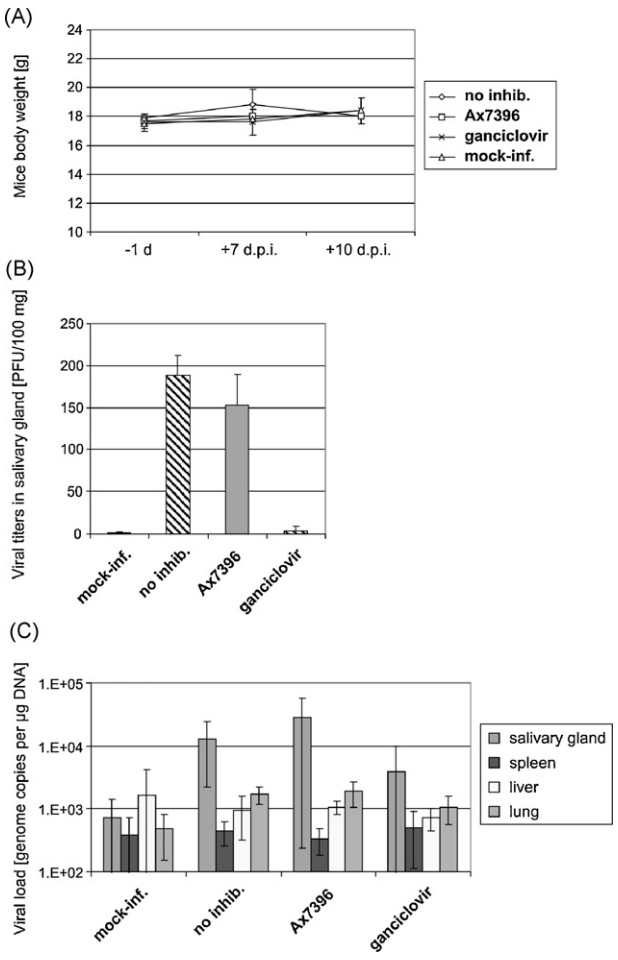

Fig. 7. Mouse model: analysis of body weight, infectious viral titers and genomic viral load. Animals were infected with MCMV and treated with antiviral drugs (days −1, 1, 4 and 7) according to the procedures described in the text. (A) The gain or loss of body weight was recorded on day −1 (prior infection) and days +7 and +10 post-infection. Mean data of the five animals of each group are given. (B) Viral titers in the salivary gland were dermined by analyzing tissue samples in a plaque formation assay on MEFs. A mean of four values is presented in each column. (C) Viral genomic load was determined by qPCR with DNA templates prepared from the tissues indicated. All qPCR reactions were performed in duplicate and the mean data of the five animals of each group are presented.

were negative). Under treatment with Ax7396, a slight reduction was noted but the difference between Ax7396 and the placebo group (no inhibition) was not significant (Fig. 7B). In contrast, the control with ganciclovir (GCV) showed almost complete clearance of the virus. In a next step, samples from salivary glands, spleens, livers and lungs were taken for the determination of viral genomic loads. As determined by preceding experiments, positive qPCR signals were mainly expected for salivary gland and lung but other organs were also positive at times for some animals. In this experiment, the mean of each set of 10 values (duplicate qPCR for five animals) showed that positive signals were exclusively obtained for the salivary gland. No inhibition was noted for Ax7396 and GCV showed a partial inhibitory effect (Fig. 7C). Thus, Ax7396 was not efficient in inhibiting MCMV replication in the mouse model under the chosen conditions. An explanation for the low efficacy *in vivo* might be given on the one hand by the low solubility of the compound (18 μM for Ax7396, compared to 128 μM for gefitinib) and, on the other hand, by a high level of clearance/metabolism of the drug. *In vitro* clearance was determined experimentally by the use of mouse liver microsomes (Clint assay, i.e. incubation of drugs for 50 min at 37 °C with liver microsomal extracts before determination of drug clearance/metabolism by LC/MS) resulting in a mean value of 533 μl/(min mg) for Ax7396 (data not shown).

4. Conclusions

Both quinazoline compounds analyzed in this study, gefitinib (Iressa®) and Ax7396 (RGB-315389), showed an interesting profile of anti-cytomegaloviral activity *in vitro*. With the *in vivo* models used, an antiviral effect was demonstrated for gefitinib (GPCMV-infected guinea pigs), whereas Ax7396 had no significant effect (MCMV-infected mice). The analysis of gefitinib in the guinea pig model showed moderate levels of inhibition of cytomegaloviral loads in blood and lung tissue and a clear reduction of weight loss and mortality of infected animals. Nevertheless, the promising data derived from *in vitro* analyses did not lead to a comparably successful outcome for gefitinib in the *in vivo* analysis. The *in vivo* data showed that a general antiviral block could not be achieved by gefitinib treatment. Possibly combination therapy with conventional anti-cytomegaloviral drugs, alternative routes of application (intravenous injection) or twice-daily treatment might improve its efficacy *in vivo*. Therefore, further experiments are required to clarify the drug's antiviral potential and value in antiviral drug development.

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