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RESEARCH ARTICLE

The multi-targeted kinase inhibitor sorafenib inhibits human cytomegalovirus replication

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Abstract Human cytomegalovirus (HCMV) is a major pathogen in immunocompromised individuals. Here, non-toxic concentrations of the anti-cancer kinase inhibitor sorafenib were shown to inhibit replication of different HCMV strains (including a ganciclovir-resistant strain) in different cell types. In contrast to established anti-HCMV drugs, sorafenib inhibited HCMV major immediate early promoter activity and HCMV immediate early antigen (IEA) expression. Sorafenib is known to inhibit Raf. Comparison of sorafenib with the MEK inhibitor U0126 suggested that sorafenib inhibits HCMV IEA expression through inhibition of Raf but independently of signaling through the Raf downstream kinase MEK 1/2. In concordance, siRNA-mediated depletion of Raf but not of MEK-reduced IEA expression. In conclusion, sorafenib diminished HCMV replication in clinically relevant concentrations and inhibited HCMV IEA expression, a pathophysiologically relevant event that is not affected by established anti-HCMV drugs. Moreover, we demonstrated for the first time that Raf activation is involved in HCMV IEA expression.

Keywords Human cytomegalovirus · Sorafenib · Kinase inhibitor · Raf · Immediate early antigen · Cancer chemotherapy · Oncomodulation · Antiviral therapy

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Introduction

Human cytomegalovirus (HCMV) belongs to the herpes viruses. After primary (normally non-recognized) infection, it persists life-long in the body. Infection rates vary from about 50 to 100% of populations depending on the socio-economic status and the geographical region. Although HCMV has been shown to be more or less regularly reactivated in infected people with detectable virus levels in the blood, HCMV disease is in general regarded to be an extremely rare event in immunocompetent persons [1–3]. However, HCMV appears to frequently reactivate in critically ill immunocompetent patients and then to be associated with prolonged hospitalization or death [4]. Moreover, HCMV is a prominent pathogen in immunocompromised individuals causing severe and often fatal

diseases [1, 2]. It is also a major cause of failure of stem cell transplantations in cancer patients [5]. Effective anti-HCMV drugs including ganciclovir, cidofovir, and foscarnet, are available but their use is limited by toxicity and the emergence of resistances [6, 7]. After allogeneic hematopoietic stem cell transplantation, about 60–70% of HCMV-positive patients will experience reactivation, and without ganciclovir prophylaxis or pre-emptive therapy, 20–30% of these will develop (multi-)organ disease such as pneumonia, hepatitis, gastroenteritis, retinitis, and encephalitis [5, 8]. Even after pre-emptive therapy, about 18% of patients develop late-onset HCMV disease (defined as >100 days after transplantation) with a mortality rate of nearly 50%. About 8% of late-onset HCMV disease patients develop antiviral drug resistance [9, 10]. Currently, there is no established treatment for drug-resistant viruses.

Anti-cancer treatment regimens may influence the HCMV disease course. Cancer therapies have been shown to stimulate HCMV reactivation also in the absence of immunosuppression [11–14]. On the other hand, the cytotoxic agents methotrexate and etoposide were shown to inhibit HCMV replication [15–17]. Therefore, it would be of interest to know how individual anti-cancer drugs influence HCMV reactivation and replication. This knowledge may be helpful for the design of therapies for cancer patients and also for other immunocompromised individuals at risk of HCMV disease. Possibly, HCMV-stimulating drugs could be spared and/or HCMV-inhibiting drugs may be selected.

Currently developed anti-cancer treatment strategies, the so-called "targeted therapies", intend to (more or less) specifically interfere with the abnormal cellular signaling events that characterize cancer cells [see e.g., 18, 19]. There is an overlap between oncogenic signaling events and cellular signaling involved in human cytomegalovirus replication. For example, activation of the transcription factor nuclear factor κ B (NF-κB), PI3K signaling, or MAPK/ERK signaling are known to be involved in both processes [20–26]. In this context, the mTOR inhibitor rapamycin (Sirolimus®) was shown to inhibit HCMV replication [27] and to protect transplant recipients from HCMV reactivation [see e.g., 28]. Moreover, the CDK2 inhibitor roscovitine (Seliciclib®) impaired HCMV replication [29].

Sorafenib (Nexavar®) is a multitargeted tyrosine kinase inhibitor registered for anti-cancer treatment that inhibits c-Raf (RAF1), B-Raf (BRAF), vascular endothelial growth factor receptor 1 (VEGFR1, FLT1), VEGFR2 (KDR), platelet-derived growth factor receptor β (PDGFRβ, PDGFRB), FLT-3 (FLT3), and c-KIT (KIT) [30]. Sorafenib may interfere with MAPK signaling and MAPK signaling has been found to be critical for HCMV replication [21, 22]. Moreover, the kinase inhibitors U0126, PD98059 (both by interference with MEK), imatinib (through inhibition of PDGFR), and gefitinib (by inhibition of the HCMV kinase UL97) have already been found to interfere with HCMV replication [21, 31–33].

Here, we investigated the influence of sorafenib on HCMV gene expression and replication in primary human foreskin fibroblasts (HFFs), primary human retinal pigment epithelial (RPE) cells, endothelial cells (primary human umbilical vein endothelial cells (HUVECs)), and glioma cells. Sorafenib inhibited HCMV replication in non-toxic clinically relevant concentrations in all investigated cell types. Moreover, sorafenib reduced HCMV immediate early antigen (IEA) expression, a pathophysiologically relevant event not targeted by established anti-HCMV drugs.

Materials and methods

Drugs

Sorafenib, sunitinib, imatinib, gefitinib, and U0126 were purchased from ENZO Life Sciences (Lörrach, Germany). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), also commonly known as phorbol 12-myristate 13-acetate (PMA) was obtained from Merck KgaA (Darmstadt, Germany).

Cell and virus culture

HFFs, RPE cells, and HUVECs were cultivated as described before [34–36]. MRC-5 (human lung fibroblasts) cells and the glioblastoma cell lines U373MG and T98G were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown at 37°C in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and containing 100 IU/ml of penicillin and 100 µg/ml streptomycin.

Human cytomegalovirus (HCMV) Strain Hi91 was isolated from the urine of an AIDS patient with HCMV retinitis [31]. The endotheliotropic clinical isolate VR1814 was obtained from Gabriele Hahn (Max von Pettenkofer-Institut für Virologie, Munich, Germany). The HCMV strains Towne and AD169 were received from ATCC (Manassas, VA, USA). The ganciclovir-resistant strain AD169^{r}GCV^{10} was established by stepwise adaptation of AD169 to replication in the presence of 10 µM ganciclovir.

Virus stocks were prepared in human foreskin fibroblasts maintained in minimal essential medium (MEM) with 4% fetal calf serum (FCS). The titers were determined by plaque titration as described previously [37].

Virus infectivity assay

Confluent cultures of HFFs, RPE cells, or HUVECs were incubated with HCMV at the indicated multiplicities of infection (MOI). After incubation for 1 h, which was required for virus adsorption, the cells were washed with PBS and incubated in maintenance medium containing 4% FCS. As described previously [34–36], cells producing HCMV-specific antigens were detected 24 h post infection by immunoperoxidase staining using monoclonal antibodies directed against the UL123-coded 72-kDa immediate early antigen 1 (IEA1) (DuPont, Bad Homburg, Germany) and 72 h (HFFs, T98G, U373MG) or 96 h (RPE cells, HUVECs) post-infection by immunoperoxidase staining using monoclonal antibodies directed against UL55-encoded late antigen gB (LA) (kindly provided by K. Radsak, Institut für Virologie, Marburg, Germany).

Virus yield assay

The amount of infectious virus was determined by virus yield assay in a single-cycle assay format as described before [34–36]. Virus titers were expressed as 50% of tissue culture infectious dose ($TCID_{50}$) 120 h post-infection.

Viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as described before [34–36]. Confluent HUVEC cultures in 96-well microtiter plates were incubated with culture medium containing serial dilutions of the indicated substances. After 5 days of incubation MTT (1 mg/ml) was added and after an additional 24 h cells were lysed in a buffer containing 20% (w/v) SDS and 50% *N*,*N*-dimethylformamide adjusted to pH 4.5. Absorbance at 570 nm was determined for each well using a 96-well multiscanner. After subtracting background absorbance, results are expressed as cell number compared to control cells that were maintained in the presence of solvent. The inhibitory concentration of 50% (IC_{50}) was calculated as the concentration of drug yielding 50% of dye reduction compared to untreated control.

Immunoblotting

Cells were lysed in Triton X-sample buffer and separated by SDS-PAGE, as described previously [34–36]. Proteins were detected using specific antibodies against β-actin (Sigma-Aldrich Chemie GmbH, Munich, Germany), ERK 1/2, the phosphorylated forms of ERK 1/2, MEK 1/2, the phosphorylated forms of MEK 1/2, Raf, the phosphorylated forms of Raf (Ser289/296/301), or MEK 1 (each from New England Biolabs, Frankfurt am Main, Germany) and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

Sub-G1 cells

The fraction of cells with fractional DNA content ("sub-G1" cell subpopulation) indicates cytotoxicity. Sub-G1 cells are considered to be dead (usually apoptotic) cells. Cells were fixed with 70% ethanol for 2 h at −20°C. The cellular DNA was stained using propidium iodide (20 μg/ml) and analyzed by flow cytometry (FacsCalibur, BD Biosciences, Heidelberg, Germany).

Quantification of viral mRNA

Total RNA was isolated from cell cultures using TRI reagent (Sigma-Aldrich, Munich, Germany). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed as described previously [38]. The following primers were used:

18S: forward GTG AAA CTG CGA ATG GCT CAT (Operon, Cologne, Germany), reverse CTG ACC GGG TTG GTT TTG AT (Operon), Taqman probe FAM-TGG TCG CTC GCT CCT CTC CA C-TAMRA (Applied Biosystems, Darmstadt, Germany).
UL123: forward GCC GCA CCA TGT CCA CT (Operon), reverse CAA CGA GAA CCC CGA GAA AG (Operon), Taqman probe VIC-CCT TAA TCT GTT TGA CGA GTT-TAMRA (Applied Biosystems)
UL122: forward CTC GCT ATC AGA CAA CGA GAA CCC (Operon), reverse GAT CAC GAT ACA GCG AGA AAG (Operon), Taqman probe VIC-CTC GGG CTT GAT GTC TTC CTG-TAMRA (Applied Biosystems)
UL83: forward ACG GCC GGA TTG TGG AT (Operon, Cologne, Germany), reverse GCG CCC GAA GAG GAC AC (Operon)
UL99: forward TGA GCC CCT GAA AGA TGC TC (Operon), reverse TCT GTT GCC GCT CCT CGT (Operon)

Quantification of viral DNA

Confluent RPE cells were treated with Sorafenib (2.5 μM) or DMSO for 24 h. Then, cells were infected for 2 h with HCMV (Towne or VR1814) at an MOI of 1 PFU/cell. This was followed by a short citrate wash to inactivate non-internalized virions. At 6 and 72 h after infection, nuclei or whole cell extracts were prepared, respectively, and DNA was purified using the DNeasy Blood & Tissue Kit

(Qiagen, Hilden, Germany). Viral DNA quantification was carried out by real-time PCR using the LightCycler Fast Start DNA Master^{PLUS} SYBR Green I Kit from Roche (Mannheim, Germany) according to the manufacturer's instructions. The following primer sequences were used for the PCRs: UL54-P Fw and Rv (described in [39]) as well as TUBB-T Fw (5′-TATCAGCAGTACCAGGATGC-3′) and TUBB-T Rv (5′-TGAGAAGCCTGAGGTGATG-3′). The identities of the PCR products were verified by melting curve analysis. DNA levels were calculated using the efficiency-corrected relative quantification strategy described in Roche Applied Science Technical Note no. LC 13/2001 (http://www.gene-quantification.de/roche-rel-quant.pdf).

Luciferase reporter assays

Human fibroblasts (MRC-5) were plated in 12-well dishes at a density of 1×10^5 cells per well and cultured for approximately 24 h in antibiotic-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS. At 1 h prior to transfection, the culture supernatants were replaced with Opti-MEM I Reduced Serum Medium (Invitrogen). Cell monolayers (≥90% confluent) were transfected using Lipofectamine 2000 (Invitrogen) and 1.6 µg of plasmid pGL3-MIEP in Opti-MEM I Reduced Serum Medium according to the manufacturer's instructions. pGL3-MIEP contains the HCMV (Towne) major immediate-early promoter/enhancer (750 bp upstream and 7 bp downstream relative to the transcription start site) attached to the firefly luciferase coding sequence and has been described previously [39]. At 4–6 h following transfection, the medium was replaced with DMEM containing 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and the respective kinase inhibitors or DMSO. At 24 h after transfection, the culture supernatants were replaced once again with fresh medium (DMEM, 10% FCS, penicillin, streptomycin, kinase inhibitors/DMSO). Finally, cells were lysed and luciferase assays were performed 48 h post transfection using the Luciferase Assay System from Promega (Mannheim, Germany) exactly following the instructions provided by the manufacturer. Relative light units were normalized to total protein content as determined by Bradford assay (Bio-Rad, Munich, Germany).

RNA interference experiments

The synthetic siRNA oligonucleotides [ON-TARGETplus SMARTpool siRNAs targeting RAF1 (encodes Raf, NM_002880) and MAP2K1 (encodes MEK 1, NM_002755)] were purchased from Dharmacon (Lafayette, CO, USA). The non-target siRNA ON-TARGETplus SMARTpool (Dharmacon) was used as negative control. Transfections were performed using the Neon^{TM} Transfection System (Invitrogen) according the manufacturer's protocol. HFFs were grown to about 60–80% confluency. Then, HFFs were trypsinized and 1×10^6 cells were resuspended in 100 µl resuspension buffer added with 2.5 µM siRNA. Electroporation was performed in a pipette tip chamber with previously optimized adjustments (voltage 1,500, width 20, pulses2). After electroporation, cells were transferred in pre-warmed IMDM plus 10% FCS. Gene expression analysis and infection experiments were performed 48 h post-infection.

Results

Influence of sorafenib on HCMV replication in human foreskin fibroblasts

Human cytomegalovirus genes are classified in immediate early, delayed early, and late genes depending on their expression during the virus replication cycle. Immediate early genes are transcribed immediately after infection and do not depend on synthesis of viral DNA or transcription of proteins. Delayed early proteins are represented by the viral DNA polymerase and other viral functions required for viral DNA synthesis and some viral structural proteins. Late genes encode mostly structural proteins used in viral assembly and packaging, and are generally expressed subsequent to delayed early genes [40].

If not otherwise stated, sorafenib was continuously present in cell culture media starting with a 24-h pre-incubation period prior to infection. HFFs were infected with HCMV strain Hi91 at MOI 0.1. Dose–response curves for the effects of sorafenib on HCMV IEA (24 h post-infection) and LA (72 h post-infection) expression in HFFs are shown in Fig. 1a. Dose–response curves for their effects on HFF viability (determined after 120 h treatment) are given in Fig. 1b. The concentrations that inhibited 50% of HCMV antigen expression (IC_{50}) or cell viability (CC_{50}) are presented in Table 1. Since LA expression has been shown to be indicative for HCMV replication [34–36], the therapeutic index (TI) was defined as CC_{50}/IC_{50} LA expression. Sorafenib inhibited HCMV antigen expression in non-toxic concentrations resulting in a TI of 15.00. While sorafenib (2.5 µM) was found not to affect HFF viability, HCMV LA expression was completely suppressed in this concentration. Similar results were received for sorafenib with the HCMV isolate VR1814 (IC_{50} LA expression = 0.51 ± 0.16 µM; TI = 10.88).

Moreover, 2.5 µM sorafenib completely suppressed formation of HCMV-induced cytopathogenic effects (CPE) detected 120 h post-infection in HFF (not shown) and

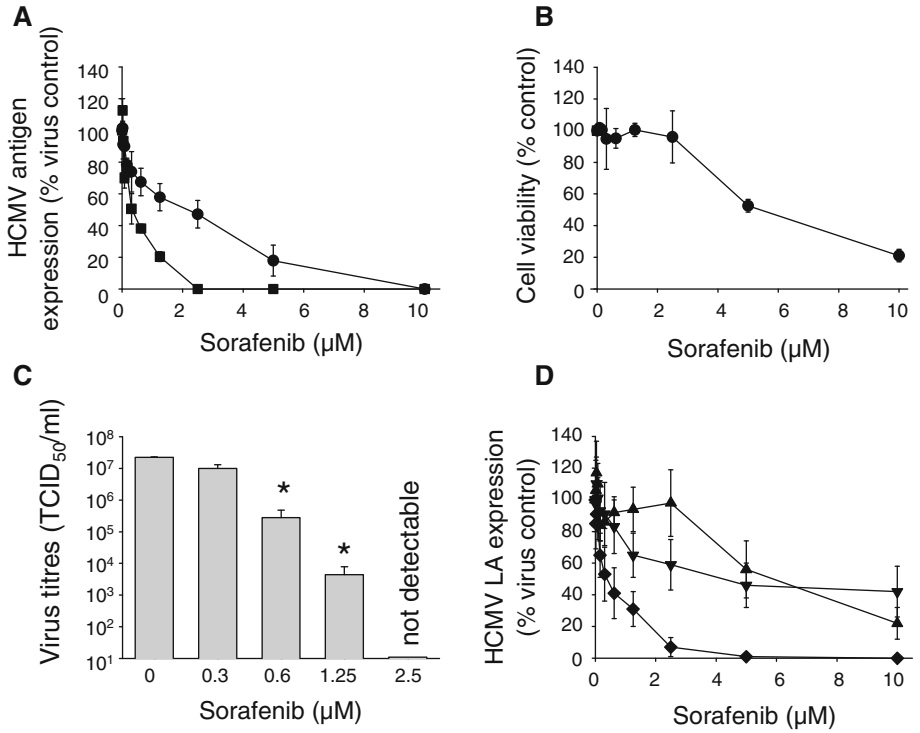

Fig. 1 Influence of sorafenib on HCMV replication in human foreskin fibroblasts (HFFs). **a** HCMV strain Hi91 immediate early antigen (IEA, determined 24 h post-infection, *filled circle*) or late antigen (LA, determined 72 h post-infection, *filled square*) expression in MOI 0.1-infected HFFs in the absence or presence of sorafenib (drug was continuously present starting with a 24-h pre-incubation period) as determined by immunostaining. **b** Influence of sorafenib on HFF viability determined by MTT assay after a 120-h treatment period. **c** Influence of sorafenib on viral titers (expressed as $TCID_{50}$/ml) determined 120 h post-infection (sorafenib was continuously present starting with a 24-h pre-incubation period) in HCMV strain Hi91 (MOI 0.1)-infected HFFs; $*p < 0.05$ relative to non-treated virus control. **d** Dose–response curves showing the influence of different sorafenib treatment schedules on HCMV LA expression as indicated by immunostaining. HCMV strain Hi91 (MOI 0.1)-infected HFFs were treated with sorafenib 24 h prior to infection (*filled triangle*), during the 1-h infection period (*filled down pointing triangle*), or after wash-out of input virus (*filled diamond*)

Table 1 Sorafenib concentrations that inhibit 50% of HCMV immediate early antigen (IEA) or late antigen (LA) expression (IC_{50}), 50% of cell viability (CC_{50}) and therapeutic indices (TI = CC_{50}/IC_{50} LA expression)

	Sorafenib (μM)			
	IEA IC_{50}	LA IC_{50}	CC_{50}	TI
HFFs	1.03 ± 0.32[a]	0.37 ± 0.08	5.55 ± 0.61	15.00
RPE cells	1.15 ± 0.16	0.58 ± 0.09	5.79 ± 0.82	9.98
HUVECs	0.61 ± 0.12	0.41 ± 0.08	3.16 ± 0.52	6.08
U373MG	1.96 ± 0.38	0.86 ± 0.23	13.27 ± 2.24	15.43
T98G	2.24 ± 0.69	0.84 ± 0.31	10.76 ± 1.51	12.81

All cell types were infected with strain Hi91 (human foreskin fibroblasts (HFFs), MOI 0.1; retinal pigment epithelial (RPE) cells, MOI 10, U373MG, MOI 1; T98G, MOI 1) except human umbilical vein endothelial (HUVECs) that were infected with strain VR1814 (MOI 10). In HFFs, RPE cells, and HUVECs, sorafenib was continuously present starting with a 24-h pre-incubation period. In the glioblastoma cell lines U373MG and T98G, sorafenib was added after virus adsorption

[a] Values are mean ± SD

inhibited HCMV DNA synthesis (25 ± 2.5 fold decreased HCMV DNA levels in sorafenib (2.5 μM)-treated HFFs vs. non-treated virus controls 72 h post-infection) but did not affect viral input DNA accumulation in the nucleus (relative DNA levels of sorafenib (2.5 μM)-treated HFFs vs. non-treated virus controls 1.2 ± 0.8-fold).

Although HCMV LA expression clearly reflects HCMV replication [34–36], the influence of non-toxic sorafenib concentrations on HCMV replication was next confirmed by virus yield assay (Fig. 1c). Sorafenib (2.5 μM) was chosen as the highest non-toxic concentration. Determination of the number of sub-G1-cells confirmed that this sorafenib concentration did not affect HFF cell viability. The fraction of sub-G1 cells remained below 5% in non-treated as well as in sorafenib (2.5 μM)-treated fibroblasts after 120 h. Treatment of HCMV Hi91 (MOI 0.1)-infected cells with sorafenib (2.5 μM) reduced HCMV titers to non-detectable levels. Also, sorafenib (2.5 μM) treatment of HCMV Hi91 MOI 1 or MOI 10-infected HFFs resulted in complete suppression of

Table 2 Sorafenib concentrations that inhibit 50% of human cytomegalovirus (HCMV) immediate early antigen (IEA) or late antigen (LA) expression (IC_{50}) in different cell types as determined by immunostaining after treatment by different application schedules

	Sorafenib IC_{50} (μM)					
	24-h pre-treatment[a]		Addition during virus adsorption[a]		Treatment post-infection[a]	
	IEA	LA	IEA	LA	IEA	LA
HFFs[b]	7.51 ± 1.31	6.42 ± 0.78	>10	4.63 ± 0.92	1.42 ± 0.13	0.41 ± 0.06
RPE cells[b]	5.66 ± 0.89	4.54 ± 1.12	3.43 ± 0.31	3.32 ± 0.52	1.04 ± 0.09	0.84 ± 0.15
HUVECs[b]	>10	9.15 ± 2.17	2.48 ± 0.22	7.88 ± 1.98	0.38 ± 0.06	0.90 ± 0.11

[a] Cells were infected with HCMV for 1 h. Then virus-containing medium was replaced by fresh virus-free cell culture medium. "24-h pre-treatment" means that cells were incubated with sorafenib for 24 h prior to virus infection; "addition during virus adsorption" means that sorafenib was only present during the 1-h infection period; "treatment post-infection" means that sorafenib was added after the adsorption period

[b] HFFs were infected with HCMV strain Hi91 at MOI 0.1, RPE cells with HCMV strain Hi91 at MOI 10, HUVECs with the endotheliotropic HCMV isolate VR1814 at MOI 10

virus replication (data not shown) indicating that the anti-HCMV activity of sorafenib does not in principle depend on the amount of infectious virus.

Finally, time-of-addition experiments were performed in order to see at which point(s) of the HCMV replication cycle sorafenib might act. HFFs were either treated with sorafenib for 24 h prior to infection, sorafenib was added solely during the 1-h adsorption period, or sorafenib was added after the wash-out of input virus. Concentration-dependent effects of these treatment schedules on HCMV LA expression are shown in Fig. 1d. Pre-treatment for 24 h or treatment during the adsorption period did not significantly affect HCMV IEA or LA expression in non-toxic concentrations whereas virus addition after the wash-out of input virus caused similar antiviral effects like those obtained for continuous treatment starting with a 24-h pre-incubation period (Tables 1, 2). These observations suggest that sorafenib efficiently blocks one or more post-entry events in the HCMV replication cycle.

Influence of sorafenib on ganciclovir-resistant HCMV

Since the molecular mechanism that underlies the anti-HCMV activity of sorafenib appears to differ from that of established anti-HCMV drugs (ganciclovir, foscarnet, cidofovir) that target the viral DNA polymerase [6, 7], we investigated the influence of sorafenib on the replication of the ganciclovir-resistant HCMV strain $AD169^{r}GCV^{10}$. $AD169^{r}GCV^{10}$ was established by stepwise adaptation of the HCMV strain AD169 to growth in the presence of ganciclovir (10 μM). The ganciclovir IC_{50} value was 2.36 ± 0.61 μM for AD169 and 83.66 ± 9.79 μM for $AD169^{r}GCV^{10}$. Sorafenib inhibited $AD169^{r}GCV^{10}$ antigen expression and replication in non-toxic concentrations (IC_{50} IEA = 1.16 ± 0.35 μM; IC_{50} LA = 0.64 ± 0.22 μM; TI = 8.67).

Influence of sorafenib on HCMV replication in retinal pigment epithelial cells and human umbilical vein endothelial cells

The influence of agents on HCMV replication may differ between different cell types. While NF-κB inhibition is known to inhibit HCMV replication in HFFs [23, 24], NF-κB inhibitors do not impair HCMV replication in RPE cells [31]. Therefore, we investigated the influence of sorafenib on HCMV replication also in RPE cells and in HUVECs. Sorafenib inhibited HCMV strain Hi91 replication in RPE cells in a similar manner as seen in HFFs (Table 1; Fig. 2). Representative pictures of HCMV IEA and LA expression in RPE cells in the presence or absence of a non-toxic concentration of sorafenib (2.5 μM) are presented in Suppl. Figure 1. Time-of-addition experiments indicated that 24-h pre-treatment or treatment during the 1-h virus adsorption period did not substantially affect HCMV replication in RPE cells in non-toxic concentrations but that sorafenib attenuates HCMV replication when added after wash out of input virus (Fig. 2c).

Moreover, sorafenib inhibited replication of the endotheliotropic HCMV isolate VR1814 in HUVECs (Table 1; Fig. 2). Again, time-of-addition experiments showed that the presence of sorafenib after wash out of input virus was critical for antiviral effects (Fig. 2d).

Influence of sorafenib on HCMV replication in glioblastoma cells

Human cytomegalovirus has been suggested to infect cancer cells and to increase cancer cell malignancy [3]. Most clinical evidence about the interaction of HCMV with cancer cells results from glioblastoma studies. Here, the influence of sorafenib was studied on HCMV replication in the glioblastoma cell lines U373MG and T98G (Table 1; Fig. 3),

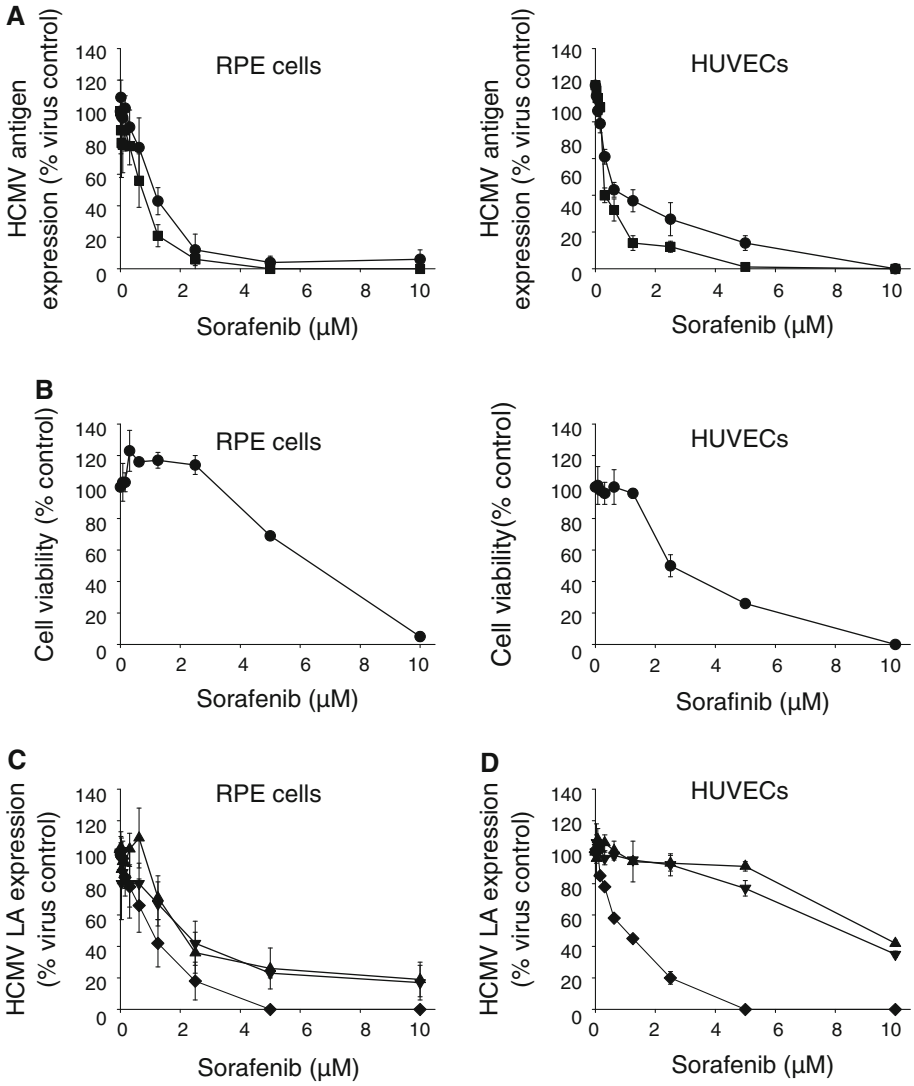

Fig. 2 Influence of sorafenib on HCMV replication in retinal pigment epithelial (RPE) cells and human umbilical vein endothelial cells (HUVECs). **a** HCMV strain Hi91 (RPE cells) or VR1814 (HUVECs) immediate early antigen (IEA, determined 24 h post-infection, *filled circle*) or late antigen (LA, determined 120 h post-infection, *filled square*) expression in MOI 10-infected RPE cells or HUVECs in the absence or presence of sorafenib (the drug was continuously present starting with a 24-h pre-incubation period) as determined by immunostaining. **b** Influence of sorafenib on RPE cell or HUVEC viability determined by MTT assay after a 120-h treatment period. **c** Dose–response curves showing the influence of different sorafenib treatment schedules on HCMV LA expression as indicated by immunostaining. HCMV strain Hi91 (MOI 10)-infected RPE cells or VR1814 (MOI 10)-infected HUVECs were treated with sorafenib 24 h prior to infection (*filled triangle*), during the 1-h infection period (*filled down pointing triangle*), or after wash-out of input virus (*filled diamond*)

that have been previously used to study effects of HCMV on glioblastoma [41, 42]. Glioblastoma cells were infected with HCMV strain Hi91 at MOI 1. Sorafenib was added after virus adsorption and inhibited both HCMV IEA and LA expression in non-toxic concentrations (Table 1).

Influence of sorafenib on HCMV gene expression

HFFs were infected with HCMV strain Hi91 at MOI 1. Expression of the HCMV immediate early genes UL123 (encodes immediate early antigen 1) and UL122 (encodes immediate early antigen 2) was determined 24 h post-infection by qRT-PCR. Expression of the delayed early HCMV gene UL83 (encodes phosphoprotein pp65) and the late HCMV gene UL99 (encodes phosphoprotein pp28) was investigated 72 h post-infection by qRT-PCR. Sorafenib (1.25 or 2.5 μM) treatment significantly reduced expression of all four investigated genes in a concentration-dependent manner (Suppl. Figure 2).

Influence of sorafenib on activity of the HCMV major immediate early promoter and contribution of immediate early antigen expression inhibition to the anti-HCMV effects of sorafenib

In contrast to the established anti-HCMV drugs ganciclovir, foscarnet, and cidofovir that interfere with the HCMV DNA polymerase but do not affect IEA expression [40, 43], sorafenib significantly reduced IEA expression (Figs. 1, 2, 3; Tables 1, 2; Suppl. Figure 2). In concordance with its inhibitory effects on HCMV IEA expression sorafenib also inhibited HCMV MIEP activity in fibroblasts as determined by luciferase assay (Fig. 4).

In this context, the question was also addressed whether the sorafenib-induced inhibition of HCMV replication may be the sole consequence of interference with major immediate early promoter (MIEP) activity and HCMV IEA expression or whether sorafenib may also target later points of the HCMV replication cycle. Sorafenib was added to

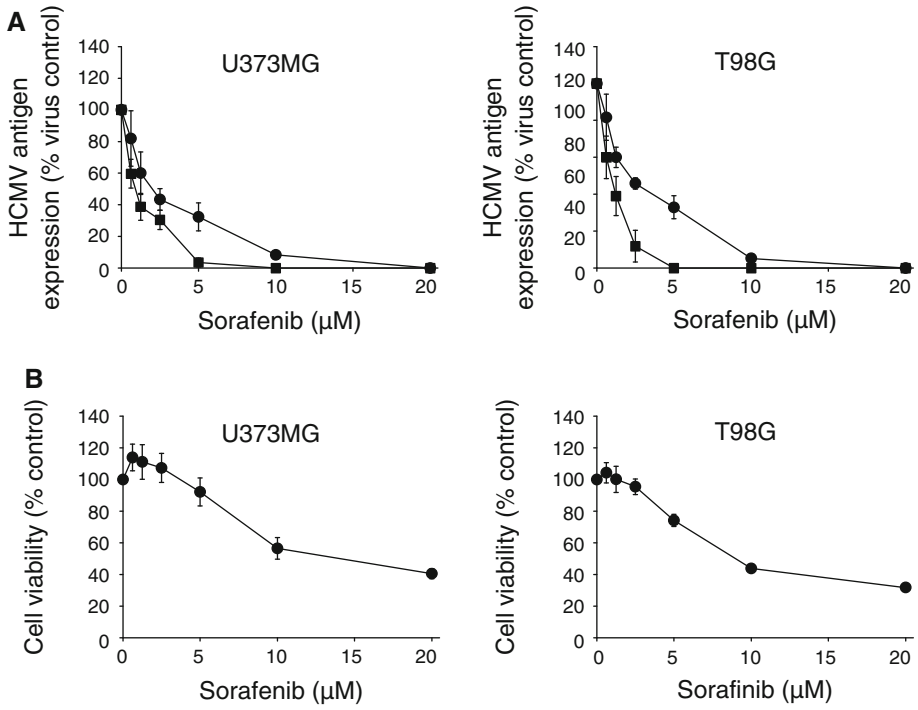

Fig. 3 Influence of sorafenib on HCMV replication in glioblastoma cells. **a** HCMV strain Hi91 immediate early antigen (IEA, determined 24 h post-infection, *filled circle*) or late antigen (LA, determined 72 h post-infection, *filled square*) expression in MOI 1-infected U373MG or T98G cells in the absence or presence of sorafenib (the drug was added after the adsorption period) as determined by immunostaining. **b** Influence of sorafenib on U373MG or T98G cell viability determined by MTT assay after a 72-h treatment period

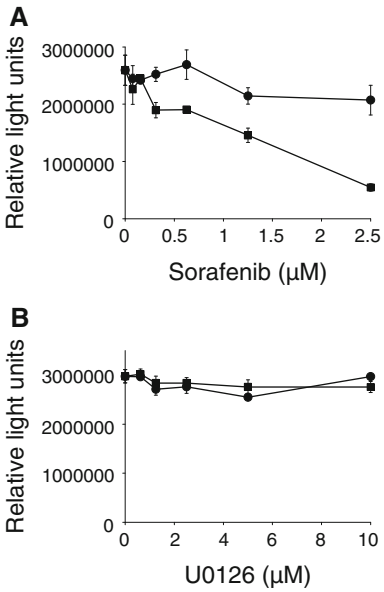

Fig. 4 Influence of sorafenib on HCMV major immediate early promoter activity as indicated by luciferase assay after a 48-h treatment period

HCMV Hi91 (MOI 0.1)-infected HFFs 1, 3, 6, or 24 h after infection. Results revealed that the sorafenib IC_{50} values for inhibition of HCMV IEA and LA expression increased when sorafenib was added at later time-points (Suppl. Table 1). However, sorafenib addition 24 h post-infection, a time point at which the HCMV IEA expression phase is considered to be finished in fibroblasts [10, 40], still significantly affected HCMV LA expression (IC_{50} LA expression $= 1.34 \pm 0.37$). Moreover, sorafenib 2.5 μM was still able to completely suppress HCMV Hi91 replication in HFFs when added 24 h post-infection. Together, these data indicate that sorafenib interferes with HCMV replication both through interference with HCMV MIEP activity and IEA expression as well as through interference with later stages of virus replication.

Raf/MEK/ERK signaling in HCMV-infected human foreskin fibroblasts (HFFs)

Sorafenib inhibits Raf, the upstream MAPK kinase kinase (MAPKKK) of the MAPKs ERK in the "classical" MAPK cascade Raf (MAPK kinase kinase)/MEK (MAPK kinases)/ERK (MAPKs) in low nanomolar concentrations [30]. ERK phosphorylation has been found to be critical for HCMV replication [20–22] and infection of HFFs with HCMV strain Hi91 (MOI 0.1) resulted in increased phosphorylation of Raf, MEK, and ERK (Fig. 5a). Non-toxic concentrations of sorafenib decreased ERK phosphorylation in HFFs (Fig. 5b) and U373MG cells (Suppl. Figure 3).

If the anti-HCMV effects exerted by sorafenib depended on its interference with Raf/MEK/ERK signaling, other substances that directly interfere with this cascade should have similar effects. However, the experimentally used MEK inhibitor U0126 inhibited HCMV LA expression in Hi91 (MOI 0.1)-infected HFFs but did not significantly interfere with HCMV IEA expression as

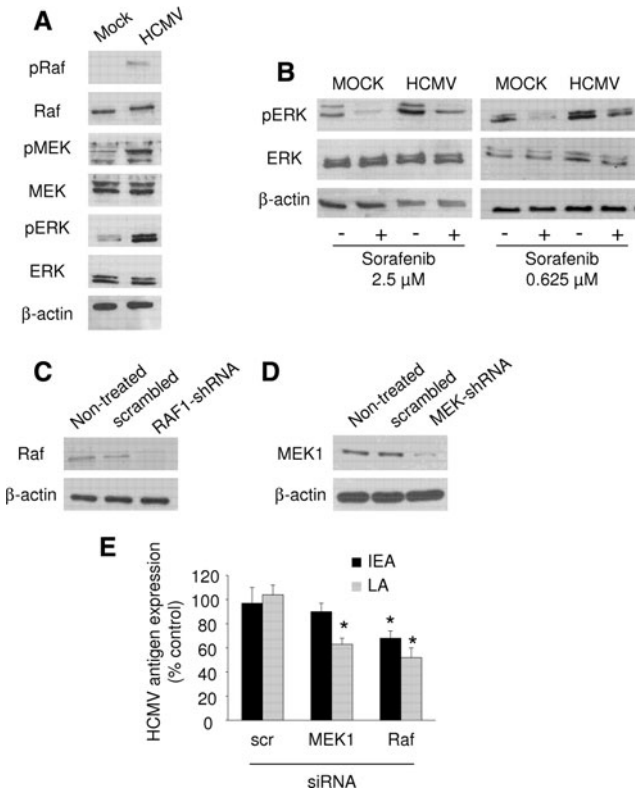

Fig. 5 Raf/MEK/ERK signaling in HCMV-infected human foreskin fibroblasts (HFFs). **a** HFFs were infected with HCMV strain Hi91 (MOI 0.1) and protein levels of Raf, phosphorylated Raf (pRaf), MEK 1/2, phosphorylated MEK 1/2 (pMEK), ERK 1/2, or phosphorylated ERK 1/2 (pERK) were determined 30 min post-infection (p.i.) by Western blot. **b** HFFs were infected with HCMV strain Hi91 (MOI 0.1) in the presence or absence of sorafenib and protein levels of ERK 1/2 or pERK were determined 30 min post-infection (p.i.) by Western blot. Non-toxic sorafenib concentrations were continuously present starting with a 24-h incubation period prior to infection. **c** HFFs were transfected with siRNA directed against Raf or non-targeted (scrambled) siRNA and Raf expression was determined 24 h post-transfection by Western blot. **d** HFFs were transfected with siRNA directed against MEK1 or non-targeted (scrambled) siRNA and MEK1 expression was determined 24 h post-transfection by Western blot. **e** Immediate early antigen (IEA) or late antigen (LA) in HCMV Hi91 (MOI 0.1)-infected HFFs that were transfected with siRNA targeting Raf or MEK1 or scrambled (scr) 48 h prior to infection

determined by immunostaining (IC_{50} IEA: > 10 μM; IC_{50} LA: 3.78 ± 0.26 μM; CC_{50}: 9.47 ± 1.20 μM; TI: 2.51). In concordance, U0126 interfered at the mRNA level with expression of the delayed early HCMV gene UL83 and of the late HCMV gene UL99 but not with the expression of the immediate early antigen-encoding genes UL123 and UL122 (Suppl. Figure 2). Moreover, U0126 did not affect HCMV MIEP activity (Fig. 4). Therefore, the sorafenib-induced inhibition of HCMV IEA expression does not appear to be consequence of interference with MEK activity.

To further examine the roles of Raf and MEK for HCMV IEA expression, expression of Raf or MEK 1 (MEK 1 is regarded to be the key mediator for ERK phosphorylation downstream of Ras/Raf [44, 45]) was inhibited by specific siRNAs prior to HCMV infection. SiRNA-mediated depletion of Raf but not of MEK 1 decreased HCMV IEA expression whereas HCMV LA expression was reduced by depletion of both kinases (Fig. 5c–e).

Discussion

Here, the influence of the anti-cancer multi-targeted tyrosine kinase inhibitor sorafenib on HCMV gene expression and replication was investigated. Sorafenib interfered with HCMV replication in non-toxic concentrations in HFFs. It inhibited HCMV MIEP activity, HCMV IEA and HCMV LA expression as well as HCMV DNA replication and reduced virus titers. Antiviral effects of substances on HCMV replication may differ in different cell types. For example, NF-κB inhibitors interfere with HCMV replication in fibroblasts [23, 24] but not in RPE cells [31]. Sorafenib exerted profound anti-HCMV effects in all tested cell types, i.e., primary HFFs, primary human RPE cells, primary HUVECs, and glioma cells, indicating that the anti-HCMV effects of sorafenib are not limited to a certain cell type.

Sorafenib inhibits more than 15 kinases in nanomolar concentrations [46]. Given this high promiscuity in the spectrum of affected cellular kinases, it remains difficult to clearly define the molecular basis of the sorafenib-caused anti-HCMV effects. Notably, none of the anti-cancer tyrosine kinases sunitinib, imatinib, dasatinib, or nilotinib inhibited HCMV replication in non-toxic concentrations (Suppl Table 1), although especially the overlap between sunitinib and sorafenib in the spectrum of affected kinases is high [30, 46]. Different indications suggest that the mechanisms by which sorafenib interferes with HCMV replication may include inhibition of Raf. The most apparent difference between sorafenib and the other investigated kinase inhibitors is that sorafenib inhibits the activity of the serine/threonine kinase Raf at low nanomolar concentrations (IC_{50} = 6 nM) [30, 47]. Cellular signaling involving the Raf downstream kinases MEK/ERK is regarded to contribute to HCMV replication by IE1 and IE2 phosphorylation [20–22]. While sorafenib inhibited HCMV-induced ERK phosphorylation in non-toxic concentrations sunitinib did not (Suppl. Figure 4). The structurally different MEK inhibitors U0126 and PD98059 had both previously been shown to suppress IE1 and IE2 phosphorylation leading to inhibition of HCMV delayed early gene expression and HCMV replication [21, 22].

However, they did not affect HCMV MIEP activity or IE1 and IE2 protein levels [21, 22]. In concordance with these previous findings, U0126 inhibited LA and delayed early gene expression but not IEA expression in the experiments presented here. These data suggest that sorafenib interferes with HCMV MIEP activity and IEA expression by mechanisms independent from effects on MEK/ERK signaling, probably by (involvement of) inhibition of Raf (Suppl. Figure 5). In concordance, siRNA-mediated depletion of Raf but not of MEK 1 that is regarded to be the key mediator for ERK phosphorylation downstream of Ras/Raf [44, 45] inhibited HCMV IEA expression. Notably, a role of Raf within HCMV IEA expression has not been described previously. However, although the assumption that sorafenib inhibits HCMV IEA expression and replication through Raf inhibition appears plausible additional research has to be performed in order to confirm (to which extent) Raf inhibition really contributes to the described anti-HCMV effects.

Human cytomegalovirus immediate early gene products exert immunomodulatory and pro-inflammatory effects and may cause pathophysiological insults also in the absence of HCMV replication [40, 43]. Moreover, immediate early gene expression is regarded to be involved in HCMV reactivation from latency [1, 40, 43]. The clinically available anti-HCMV drugs ganciclovir, cidofovir, and foscarnet that all target HCMV DNA polymerase do not affect HCMV immediate early gene expression [40, 43]. Consequently, sorafenib targets pathophysiological events in the HCMV replication cycle that are not covered by established treatment regimens. In addition, cytotoxic adverse effects and the emergence of resistances against anti-HCMV drugs remain a clinical problem and the search for alternative anti-HCMV drugs continues [6, 7]. In the context of resistances, sorafenib was also active against a ganciclovir-resistant HCMV strain and may therefore represent an option for treatment of drug-resistant HCMV strains.

PDGFR activation was reported to be critical for HCMV replication [33]. However, the mechanism by which sorafenib interferes with HCMV replication in our models does not appear to include inhibition of PDGFR for two reasons: (1) Although sorafenib concentrations that inhibit PDGFR tyrosine kinase activity ($IC_{50} = 57$ nM) are about sevenfold higher than effective sunitinib concentrations ($IC_{50} = 8$ nM) [30, 47] only sorafenib inhibited HCMV replication in primary cultures from three different cell types (HFFs, RPE cells, HUVECs) in non-toxic concentrations whereas sunitinib did not (Suppl. Tables 2, 3). (2) Imatinib, an anti-cancer kinase inhibitor that targets PDGFR and that had been previously reported to interfere with HCMV replication in nanomolar concentrations [33], and two further kinase inhibitors that target PDGFR (nilotinib, dasatinib) did not inhibit HCMV strain Hi91 replication in HFFs in non-toxic concentrations in our experiments (Suppl. Table 2). Therefore, the role of PDGFR activation for HCMV replication may vary in different experimental systems using different virus strains and cells. In a recent retrospective analysis of the first 100 days after transplantation in a cohort of hematopoietic cell transplant recipients, imatinib did not appear to reduce cytomegalovirus reactivation [48].

In addition to inhibition of cellular kinases, sorafenib might also interfere with viral kinases. The anti-cancer kinase inhibitor gefitinib had been shown to reduce HCMV replication by interference with phosphorylation of the HCMV gene product UL97, a serine/threonine kinase that phosphorylates viral and cellular protein substrates, which affect viral replication at many levels [32]. Therefore, the antiviral mechanism of sorafenib may involve effects on viral kinases like the UL97 kinase in addition to cellular kinases.

The mean through sorafenib plasma concentration was determined to be about 9 μM. Peak plasma concentrations were reported to be about 13 μM [49]. Therefore, the non-toxic sorafenib concentrations that inhibited HCMV replication being $\leq$2.5 μM with IC_{50} values for inhibition of HCMV late antigen expression <1 μM are clearly in the range of therapeutic plasma levels. Notably, sorafenib was most recently reported to inhibit hepatitis C virus (HCV) replication through interference with Raf [50]. Anti-HCV effective concentrations of sorafenib were in the range of 10–15 μM, i.e., 10–20-fold higher than anti-HCMV effective concentrations.

In addition to the role of HCMV disease in immunocompromised (cancer) patients, HCMV has been hypothesized to exert oncomodulatory effects, i.e., to infect established cancer cells and to enhance their malignancy [3, 51]. After initial experimental findings in HCMV-infected glioblastoma cells [41], HCMV has been detected in tumor tissues of glioma patients by several independent groups [2, 13, 52–55]. Remarkably, HCMV infection of gliomas was correlated with reduced patients' survival [2] or with higher tumor grades [54]. Moreover, HCMV infection of glioma cells was correlated with telomerase activation in patients' tissues [55]. Primary experimental findings suggested that oncomodulation by HCMV might be reversed by treatment with anti-HCMV drugs [56]. In the meantime, clinical trials that investigate anti-HCMV treatment for glioma patients with HCMV-infected tumors were started [2, 13]. First findings have been announced to be encouraging [57]. However, scientific publication of results is still pending. In the context of HCMV oncomodulation, the finding that sorafenib inhibits HCMV replication in glioblastoma cells might favor the use of sorafenib for patients with HCMV-infected tumors.

In conclusion, we present evidence that the clinically approved multitargeted anti-cancer kinase inhibitors sorafenib impairs HCMV replication in clinically relevant non-toxic concentrations. These data may favor the use of sorafenib cancer patients at risk of HCMV disease. Clinical monitoring of HCMV reactivation and replication in sorafenib-treated cancer patients is warranted. Moreover, our data suggest that Raf activation is involved HCMV IEA antigen expression, a previously unrecognized role of Raf during HCMV infection.

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