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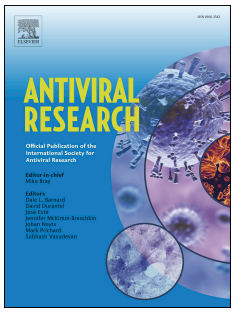

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Inhibitors of dual-specificity tyrosine phosphorylation-regulated kinases (DYRK) exert a strong anti-herpesviral activity

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Abstract

Infection with human cytomegalovirus (HCMV) is a serious medical problem, particularly in immunocompromised individuals and neonates. The success of (val)ganciclovir therapy is hampered by low drug compatibility and induction of viral resistance. A novel strategy of antiviral treatment is based on the exploitation of cell-directed signaling, e. g. pathways with a known relevance for carcinogenesis and tumor drug development. Here we describe a principle for putative antiviral drugs based on targeting dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs). DYRKs constitute an evolutionarily conserved family of protein kinases with key roles in the control of cell proliferation and differentiation. Members of the DYRK family are capable of phosphorylating a number of substrate proteins, including regulators of the cell cycle, e.g. DYRK1B can induce cell cycle arrest, a critical step for the regulation of HCMV replication. Here we provide first evidence for a critical role of DYRKs during viral replication and the high antiviral potential of DYRK inhibitors (Harmine, AZ-191, SC84227, SC97202 and SC97208). Using established replication assays for laboratory and clinically relevant strains of HCMV, concentration-dependent profiles of inhibition were obtained. Mean inhibitory concentrations (EC50) of 0.98 ± 0.08 µM/SC84227, 0.60 ± 0.02 µM/SC97202, 6.26 ± 1.64 µM/SC97208, 0.71 ± 0.019 µM/Harmine and 0.63 ± 0.23 µM/AZ-191 were determined with HCMV strain AD169-GFP for the infection of primary human fibroblasts. A first analysis of the mode of antiviral action suggested a block of viral replication at the early-late stage of HCMV gene expression. Moreover, rhesus macaque cytomegalovirus (RhCMV), varicella-zoster virus (VZV) and herpes simplex virus (HSV-1) showed a similarly high sensitivity to these compounds. Thus, we conclude that DYRK signaling represents a promising target pathway for the development of novel anti-herpesviral strategies.

1. Introduction

HCMV is one of the most complex pathogenic viruses causing life-long infections. In patients undergoing immunosuppression, HCMV can lead to life-threatening situations. Although various candidates for HCMV vaccine are under investigation at the preclinical and clinical levels, no vaccine has been licensed so far. The success of therapy with standard valganciclovir is hampered by low drug tolerability and, at a limited frequency, the induction of viral resistance (Dropulic and Cohen, 2010). All approved anti-cytomegaloviral drugs recognize an identical target molecule (the viral DNA polymerase) and thus commonly induce drug resistance. In order to reduce the adverse issues of resistance and side-effects, alternative drug candidates are needed, leading to a continued search for suitable viral and cellular target proteins. The viral terminase represents such a novel antiviral target. Its inhibition blocks viral genome processing and encapsidation, a so far unexploited step in HCMV replication (Goldner et al., 2011). Recently, the drug candidate letermovir (AIC246), which inhibits the large terminase subunit pUL56, has been announced to be successful in phase III prophylaxis clinical trials (Chemaly et al., 2014; Lischka et al., 2016). Another promising strategy is based on cell-directed protein kinase inhibitors. Coevolution between cytomegaloviruses and their hosts has resulted in a complex process of virus host interaction. In particular, protein kinases are important regulators indicated by remarkable kinome alterations induced upon HCMV infection. During infection, a great number of cellular kinases is upregulated, the phosphorylation level of proteins is enhanced and various kinase-dependent signaling pathways are modulated (Steingruber et al., 2016; Hertel et al., 2007; Yurochko, 2008; Prichard, 2009; Lee and Chen, 2010; Hutterer et al., 2013). In the focus of current antiviral drug development are cyclin-dependent protein kinases (CDKs) that are functionally integrated into efficient viral gene expression and protein

modification (Zydek et al., 2010; Kuny et al., 2010; Oduro et al., 2012). Several reports describe that HCMV replication requires the activity of CDK 1, 2, 7 and 9, respectively (Hutterer et al., 2015; Kapasi and Spector, 2008; Sanchez and Spector, 2006; Tamrakar et al., 2005; Salvant et al., 1998). Immediately after infection, the CDK activity is modulated and interferes with the ordered process of the cell cycle. A cell cycle arrest is induced establishing the conditions supportive for virus replication (Wiebusch and Hagemeier, 2011; Jault et al., 1995; Salvant et al., 1998). A key regulator of cell growth, apoptosis and differentiation is the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) that belongs to an evolutionarily conserved family of protein kinases. Members are known to be activated through autophosphorylation of tyrosine residues in the activation loop and to phosphorylate their substrates on serine and threonine residues (Liu et al., 2014; Seifert et al., 2008; Walte et al., 2013). Further kinases of this family include DYRK1B, DYRK2, DYRK3, DYRK4A and DYRK4B. Several studies reported a strong overexpression of DYRK1A, and its closest member DYRK1B, in various tumors suggesting a role in carcinogenesis (Ewton et al., 2011; Gao et al., 2009; Deng et al., 2006). Currently, efforts are undertaken to assess if DYRK1A could serve as a potential therapeutic target (Radhakrishnan et al., 2016).

In the context of herpesviral infections, a functional role of DYRK-driven pathways or an upregulation of DYRKs in herpesvirus-infected cells has not been reported to date (including comprehensive analyses of gene profiling; Tirosh et al., 2015). However, our earlier studies demonstrated that other kinases linked to hedgehog signaling can be upregulated during HCMV replication in primary human fibroblasts, such as the ULK3 kinase (factor 3.9 of upregulation compared to uninfected cells; Hutterer et al., 2013, Antiviral. Res.). Interestingly, a study based on large datasets suggested that among the numerous substrates and interactors of DYRK1A, ULK3 is one of the components of its interactome (Rouillard et al., 2016). In this study, we

describe a crucial role of DYRK kinases during herpesviral replication and present novel DYRK-targeted drug candidates with *in vitro* activity against HCMV, VZV, HSV-1, and RhCMV in a range of low micromolar concentrations.

2. Materials and methods

2.1. Antiviral compounds

Antiviral drugs and kinase inhibitors used in this study were obtained from the following sources: ganciclovir (GCV, Sigma Aldrich), cidofovir (CDV, Vistide; Pharmacia & Upjohn S.A., Luxembourg), staurosporine (STP, Calbiochem), Harmine (Sigma-Aldrich), AZ-191 (Selleckchem). SC84227, SC97202, SC97208, and SC83760 were in-house synthesized by 4SC, Martinsried, Germany. The chemical structures of SC84227, SC97202 and SC97208 are presented in Fig. 2A. For use in cell culture, stock aliquots were prepared in DMSO and stored at -20 ℃.

2.2. *In vitro* kinase assay

Selectivity kinase profiling of SC84227, SC97202 and SC97208 was performed with radioisotope based kinase assays using ^{33}P-ATP at Reaction Biology Corp. (USA). Respective peptide substrates of human DYRK kinases (DYRK1/DYRK1A, RRRFRPASPLRGPPK; DYRK1B, RRRFRPASPLRGPPK; DYRK2, RRRFRPASPLRGPPK; DYRK3, RRRFRPASPLRGPPK; DYRK4, RRRFRPASPLRGPPK) were prepared at a concentration of 20 µM in base reaction buffer (20 mM Hepes [pH 7.5], 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na_3VO_4, 2 mM DTT, 1% DMSO). Human kinase DYRK1/DYRK1A,

DYRK1B, DYRK2, DYRK3, and DYRK4, respectively, was delivered into the substrate solution mix. Control compounds (staurosporine, STP; GW5074) or DYRK inhibitors in DMSO were added to the kinase reaction mixture by Acoustic technology (Echo550; nanoliter range) and inclubated for 20 minutes at room temperature. Kinase reaction was initiated by addition of ^{33}P-ATP (10 µCi/µl) to the mixture and incubated for two hours at room temperature. Subsequently, samples were spotted onto P81 ion exchange paper and kinase activity was detected by filter-binding method.

2.3. Cultured cells and viruses

Human foreskin fibroblasts (HFFs) were grown in minimal essential medium (MEM, Gibco) and Vero cells in Dulbecco minimal essential medium (DMEM, Gibco). Media were supplemented with 7.5% (vol/vol) fetal bovine serum (FCS; Sigma-Aldrich), 10 µg/ml gentamicin, and 350 µg/ml glutamine. CMV strains AD169, AD169-GFP (Marschall et al., 2000), AD169-GFP 314/GCV^{R} (Marschall et al., 2000), TB40-UL32-EGFP (Sampaio et al., 2005), Merlin-GFP (UL128+, RL132; Stanton et al., 2000), BAC213 (AD169delUL97-GFP; Marschall et al., 2005), and rhesus macaque cytomegalovirus (RhCMV) were propagated in HFFs and used for infection assays as previously described (Marschall et al., 2000). HCMV strain Merlin (UL128þ, RL132) was derived from a Merlin-BAC recombinant, pAL1120, kindly provided by Richard Stanton (Univ. of Cardiff, U.K.) (Stanton et al., 2010) Reconstituted Merlin was propagated in RPE-1 cells (kindly provided by Barry Slobedman, Univ. of Sydney, Australia) respectively in DMEM/F12+Gluta-MAX (Invitrogen) supplemented with 10% FCS and 1xPSG with one passage in MRC-5 cells to increase viral titre. The titre of virus stocks was determined by standard plaque assay. Varicella-zoster virus (VZV, strain Oka; Takahashi et al., 1974) was propagated and used for the infection of HFFs. For herpes

simplex virus type 1 (HSV-1), strain 166vVP22-GFP (Elliott and O'Hare, 1999) was propagated in Vero cells and used for the infection of HFFs.

2.4. Immunofluorescence analysis

MRC-5 cells were seeded in 6-well culture plates with underlying coverslips and inoculated with HCMV strain Merlin at a multiplicity of infection (MOI) of 0.2 pfu/cell. Mock-infected cultures were established concurrently. After two hours incubation at 37 ℃, inoculum was replaced with fresh medium. At four days post infection, cells were washed with PBS and fixed in 4% paraformaldehyde for eight minutes followed by two washes with PBS. Cells were permeabilsed with 0.2% Triton X-100 for 20 minutes at 4 ℃ followed by an additional four washes with PBS. Blocking of non-specific staining and non-specific interactions between rabbit antibodies and HCMV-derived Fc receptors was performed by 30 minutes incubation with 2% BSA and 10% human serum in PBS. Cells were incubated with mouse monoclonal anti-HCMV immediate early (IEp72) and early (pUL44) antibody cocktail (clones DDG9 and CCH2; Dako) and either rabbit polyclonal anti-human DYRK1A antibody (Abcam) or rabbit monoclonal anti-human DYRK1B antibody (Abcam) for one hour at room temperature. Cells were rinsed in washing buffer (Dako) followed by 40 minutes incubation with Alexa Fluor 594 donkey anti-mouse and 488 donkey anti-rabbit secondary antibodies (Life Technologies). Cells were rinsed again in washing buffer, covered with ProLong Gold Antifade Reagent containing DAPI (Life Technologies) and mounted on histology slides. Imaging of cells was carried out using a Nikon Eclipse E400 light microscope with a Y-FL Epi Fluorescence attachment and a DS camera control unit DS-L2, DS camera head DS-Fil (Nikon).

2.5. Western blot analysis

Western blot (Wb) analysis was performed by standard procedures as described elsewhere (Auerochs et al., 2011; Hutterer et al., 2013).

2.6. Cytotoxicity and cell proliferation assays

Assays measuring distinct parameters of cytotoxicity or cell proliferation were performed as described earlier (Milbradt et al., 2009; Hutterer et al., 2013). Lactate dehydrogenase (LDH) release assay was performed with the CytoTox 96® NonRadioactive Cytotoxicity Assay (Promega) using media samples of cells cultured for one day in the presence of antiviral compounds. Release of LDH activity was determined according to the protocol of the manufacturer. The cell proliferation assay CellTiter 96® 156 AQueous One Solution Cell Proliferation Assay (Promega) was performed in a 96-well plate format under standard conditions (Hutterer et al., 2013).

2.7. DYRK1A knockdown by siRNA transfection of virus-infected cells

HFFs were seeded in 12-well plates and cultured to □80% confluence prior to transfection with custom designed siRNA (Santa Cruz Biotechnology) targeting DYRK1A (sc-39007) or with a scrambled control siRNA (siControl-J, sc-44238; siControl-G, sc-44235). siRNA transcripts were transfected using Lipofectamine 2000 (Life Technologies) following manufacturer's protocols (Hamilton et al., 2014). One day post-transfection, cells were infected with HCMV and HSV-1, respectively. For HCMV infection experiments, AD169-GFP was used at a MOI of 0.5 for Wb analysis (three days post-infection) or at MOI of 0.01 for the GFP-based replication assay (seven days post-

infection). For HSV-1 infection experiments, HSV-1 VP22-GFP was used at very low MOI to perform GFP-based replication assay (three days post-infection).

3. Results and discussion

3.1. Upregulation of DYRK protein kinases 1A and 1B as well as DYRK-associated regulatory proteins after infection with strains of HCMV

Cellular signaling pathways are stimulating factors of HCMV replication possessing multifold regulatory importance (Fortunato et al., 2000; Marschall et al., 2011; Tandon and Mocarski, 2012; Mocarski et al., 2013). The HCMV-specific modulation of expression levels of regulatory protein kinases, in particular their upregulation in HCMV-infected fibroblasts, has been demonstrated by various approaches including transcript profiling and proteomics technologies (Hertel et al., 2007; Yurochko, 2008; Milbradt et al., 2014; Hutterer, 2013; M.M. & C.H., unpublished data). In this study we utilized HCMV strains Merlin, TB40 and AD169 for the infection of primary human fibroblasts (MRC-5 and HFFs) at a range of MOI between 0.1 and 3 (Fig. 1). Single-cell detection of proteins by confocal immunofluorescence imaging showed an intermediate level of DYRK1A and DYRK1B, both at mostly nuclear localization (Fig. 1A, panels Mock). Upon HCMV infection, the two protein kinases were upregulated (Fig. 1A, panels AD169 and Merlin). DYRK1A was localized in the nucleus and in the cytoplasm including the virion assembly complex (cVAC; Fig. 1A, Fig. S1 and Fig. S2, indicated by yellow arrows) whereas DYRK1B was exclusively present in the nucleus and strongly localized within nuclear replication compartments of infected cells as demonstrated by colocalisation with viral IE/E staining (Fig. 1A). Incubation with nonspecific rabbit polyclonal antibodies did not produce any staining of the cVAC demonstrating successful

blocking of HCMV-derived Fc receptors (data not shown). Notably, the wild-type-like viral strain Merlin induced syncytia formation, similar to previous reports by other researchers (Booth et al., 1978; Saygun et al., 2009; Shin et al., 2008), comprising strong upregulation of DYRK1A/1B expression.

An upregulation of DYRK1A/1B was confirmed by Wb analysis, indicating the modulation of expression between 24 and 72 hours post-infection (Fig. 1B, upper two panels; compare to the cascade-like increase of viral immediate early, early and late protein production in the lower three panels). In addition, DYRK signaling regulators ULK3 and Gli2 (Varjosalo et al., 2008; Maloverjan et al., 2010; Maloverjan et al., 2010; Li et al., 2011) were likewise upregulated by HCMV infection. These findings indicate that DYRK kinases may have regulatory importance for HCMV replication.

3.2. Design of DYRK inhibitors and determination of antiviral activity

On the basis of the previously identified crystal structure of DYRK1A (Alexeeva et a., 2015; Soundararajan et al., 2013), inhibitory compounds were evaluated by *in silico* docking and biochemical screening analyses. Primary hits were validated by *in vitro* kinase selectivity panels, demonstrating a pronounced activity in the low nanomolar range against DYRK1A for the compounds SC97202, SC84227 and SC97208 (< 0.10 nM, 0.19 ± 0.04 nM, 2.1 ± 0.27 nM, Table 1, upper part). Additional activities against further members of the DYRK protein kinase family were measured at lower stringency (Table. 1, lower part). IC50 values for the closest member of DYRK1A, DYRK1B, were between 0.31 ± 0.01 nM, SC97202, and 7.61 ± 0.55 nM, SC97208, whereas activity against DYRK4 was in the micromolar range between 2.43 ± 0.19 µM, SC97202, and > 10 µM, SC84227.

Our initial analyses of putative biological consequences of DYRK inhibition in cultured cells led to the identification of antiviral activity of these compounds (Hutterer et al., 2015, 40th Int. Herpesvirus Workshop, Boise, ID, USA, abstract 1.12). We then quantitated the inhibitory activity against human and animal herpesviruses. For HCMV, an established HCMV GFP-based replication assay was applied (AD169-GFP, Marschall et al., 2000) to determine mean inhibitory values in the low or sub-micromolar range, i.e. EC50 of 0.98 ± 0.08 (SC84227), 0.60 ± 0.02 (SC97202) and 6.26 ± 1.64 µM (SC97208), respectively (Fig. 2). Of note, two commercially available reference inhibitors of DYRK protein kinases, AZ-191 and Harmine (Becker and Sippl, 2011), showed similar antiviral efficacy (Fig. 2, left panels), a finding that underlines the functional relevance of DYRK activity for HCMV replication. The analysis of additional CMVs and other herpesviruses, i.e. strains of HCMV AD169, AD169-GFP 314/GCV^R (GCV-resistant), TB40-UL32-EGFP and Merlin-GFP, as well as RhCMV (strain 68-1), VZV (strain Oka) and HSV-1 (strain VP22-GFP), indicated broadness of anti-herpesviral activity at low micromolar concentrations in particular for SC97202 and SC84227, although anti-CMV activity (β-herpesviruses) was more pronounced than anti-VZV and anti-HSV-1 activities (α-herpesviruses) (Table 2). Concerning target selectivity, we cannot exclude the possibility that the compounds may exert secondary inhibitory effects on minor targets, such as additional cellular kinases. Such impact of secondary targeting might be one of the reasons explaining that individual herpesviruses show slightly different susceptibility profiles towards the compounds.

The specificity of the antiviral potential of DYRK inhibitors was supported by an evaluation of putative drug effects on cell viability and proliferation in HFFs. Cells were treated with the panel of selected novel and reference inhibitors of DYRK, and analysed in parallel using the LDH release-based cytotoxicity assay (Fig. 3A) or the MTS-based

cell proliferation assay (Fig. 3B). In both measurements, very limited effects were obtained in the range of concentrations between 0-1000 µM (24 hours short-period measurement; Fig. 3A) or 0-5 µM (3 days long-period measurement; Fig. 3B). Notable signals of LDH release were only seen with AZ-191 and SC84227 at concentrations higher than 100 µM, and some intermediate, concentration-dependent antiproliferative activity was exclusively noted for SC84227 and SC97202, thus indicating a clear demarcation from concentrations of antiviral activity.

3.3. Importance of upregulated DYRK1A expression levels for the replication of HCMV and HSV-1

The importance of DYRK activity for the individual stages of HCMV replication was analysed by using compound-treated HCMV-infected cells that were harvested at consecutive time points post-infection (Fig. 4A). When detecting viral immediate early (IE1p72), early (pUL44) and late proteins (pp28) on WBs, a clear early-late inhibitory effect was determined for all DYRK inhibitors used. While control cells (solvent DMSO-treated) showed a steady increase of viral protein production over the time points 24, 48 to 72 hours post-infection, DYRK inhibitor-treated cells showed reduced levels and delayed onset of viral expression (note that an additional putative DYRK inhibitor, SC83760, was additionally included in this analysis). A comparison with cellular proteins functionally linked with the DYRK pathway, i.e. ULK3 kinase, Gli2 transactivator and Rb tumor suppressor protein, uniformly showed upregulation by HCMV infection and down-modulation by inhibitor treatment (Fig. 4B). This finding confirms the DYRK-directed activity of these compounds. Moreover, we performed additional experimentation to further elucidate the mode of antiviral activity of the DYRK inhibitors, i.e. we analyzed whether the mechanism of viral nuclear egress was impaired by DYRK inhibitors. We specifically addressed whether DYRK inhibitors lead to a dyslocalization of the viral nuclear egress proteins pUL50 and pUL53, i.e. dissecting them from the rim of the

nuclear lamina. The results clarified that a block of the pUL50-pUL53 co-recruitment to the nuclear rim, which had been experimentally produced by CDK inhibitor R25 and viral pUL97 inhibitor maribavir (Sonntag et al., 2016), could not be similarly produced by DYRK inhibitors (data not shown). Thus, anti-HCMV activity of DYRK inhibitors is manifested by an early-late phase block in a manner independent from nuclear egress.

In order to prove that DYRK expression is a supportive regulatory element in virus-infected cells, we performed knockdown experiments using a DYRK1A-specific synthetic siRNA. For this purpose, HFFs were transiently transfected with siRNAs and subsequently infected with HCMV AD169-GFP. The onset of infection was monitored under the microscope by the inspection of GFP expression, while the success in DYRK1A knockdown and the interference with viral protein production were demonstrated by Wb analysis (Fig. 4C; note the substantial reduction of late protein expression pp28 in lane 4). Importantly, a statistically significant decrease of viral replication was produced by siDYRK1A as compared to a scrambled siControl (Fig. 4D). The result was confirmed by three independent experiments performed at high and low multiplicities of infection (i.e two replicates plus one replicate at MOIs of 0.5 or 0.01, respectively). Thus, these experiments further illustrate the importance of upregulated DYRK1A expression levels for the replication of HCMV.

Furthermore, very similar effects of DYRK-directed compounds and siRNAs were obtained in case of HSV-1 infection. All compounds used showed a strong anti-HSV-1 activity in a concentration-dependent manner (Fig. 5A; Table 2) and for most compounds (exception SC97208), high concentrations revealed an antiviral efficacy similar to the anti-herpesviral reference drugs (CDV, GCV). Also for HSV-1, an early-late pattern of inhibition of viral proteins was detected (Fig. 5B; early and late proteins pUL42 and ICP5). In addition, a partial reduction of viral immediate early protein production ICP0 was noted for most of the compounds. Finally, the use of siDYRK1A showed a

statistically significant decrease of HSV-1 replication to levels similar to HCMV replication (Fig. 5C; compare to Fig. 4D). Summarized these results demonstrate for the first time a dependence of efficient herpesviral replication on intracellular levels and activity of DYRK kinases.

4. Conclusions

In this study we analysed novel benzohydrofurane derivatives that target DYRK activity regarding their properties of anti-herpesviral activity. The conclusions drawn from these experiments are given as follows: (i) Replication of HCMV, HSV-1, VZV and RhCMV is blocked by DYRK inhibition (ii) Dependency on DYRK activity is manifested at the early-late stage of HCMV replication, (iii) DYRK1A and DYRK1B expression is upregulated in HCMV infected fibroblasts (iv) Knock-down of DYRK1A markedly impairs replication of HCMV and HSV-1. In summary, this study provides first evidence for an important regulatory role of DYRK kinases during herpesviral replication. Thus, DYRK kinases may represent novel targets of future host cell-directed anti-herpesviral strategies.

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Table 1. Selectivity profile of SC97202, SC84227 and SC97208.

Kinase	Mean inhibitory concentration, IC50 ± SD (nM)				
	SC97202	**SC84227**	**SC97208**	**Control**	
DYRK1/DYRK1A	< 0.10	0.19 ± 0.04	2.09 ± 0.27	STP	2.27
DYRK1B	0.31 ± 0.01	1.41 ± 0.18	7.61 ± 0.55	STP	0.69
DYRK2	13.80 ± 0.58	20.83 ± 1.26	36.40 ± 1.10	STP	126.90
DYRK3	23.38 ± 1.89	125.48 ± 43.57	27.99 ± 6.93	STP	24.41
DYRK4	2431.67 ± 185.77	> 10 000	3001.33 ± 25.32	GW5074	7350.00

Compounds were tested in 6-dose IC50 triplicate mode with 10-fold serial dilution starting at 10 μM. Control compound staurosporine (STP) was tested in 10-dose IC50 mode with 4-fold serial dilution starting at 20 μM. Control compound GW5074 (c-Raf inhibitor) was tested in 10-dose IC50 mode with 3-fold serial dilution starting at 20 μM. Reactions were carried out at 10 μM ATP.

Table 2. Analysis of antiviral activities of SC97202, SC84227 and SC97208

	Mean inhibitory concentration, EC50 ± SD (μM)				
	HCMV				
Inhibitor	**AD169-GFP**	**AD169**	**AD169-GFP 314/GCV^R**	**TB40-UL32-EGFP**	**Merlin-GFP**
SC97202	0.60 ± 0.02	<1.11*	0.64*	1.05*	0.59*
SC84227	0.98 ± 0.08	1.32 ± 0.41	2.46*	6.79*	2.71*
SC97208	6.26 ± 1.64	6.55*	6.26 ± 0.17	8.67 ± 0.28	6.99 ± 0.10

	Other herpesviruses		
Inhibitor	**RhCMV**	**VZV**	**HSV-1 VP22-GFP**
SC97202	<1.11*	1.60*	3.22 ± 0.40
SC84227	0.69*	8.33 ± 1.93	3.09*
SC97208	2.20*	7.23 ± 0.57	10.00 ± 0.00

[a]Viruses and cells used: HCMV, human cytomegalovirus, strain AD169-GFP (Marschall et al., 2000), AD169, AD169-GFP 314/GCV^R (Marschall et al., 2000); TB40-UL32-EGFP (Sampaio et al., 2005), Merlin-GFP (UL128+, RL132; Stanton et al., 2000), primary human fibroblasts; RhCMV, rhesus macaque cytomegalovirus, primary human fibroblasts; HSV-1, herpes simplex virus type 1, strain 166v VP22-GFP, HFF; VZV, varicella-zoster virus, strain Oka, primary human fibroblasts. The EC50 values of virus

replication were determined by GFP-based reporter assay (AD169-GFP, AD169-GFP 314/GCV^{R}, TB40 UL32-EGFP, Merlin-GFP, and HSV-1 VP22-GFP) or plaque reduction assay (AD169, RhCMV and VZV). Mean values derived from 4-fold measurements are given; EC50 values were calculated from three concentrations (including SD) or *two concentrations of the inhibitors.

Figure legends

Fig. 1. HCMV-mediated upregulation of DYRK1A and DYRK1B in fibroblasts. (A) MRC-5 grown on cover slips were infected with HCMV strain Merlin at MOI 0.2 or remained uninfected (Mock). Cells were fixed four days post-infection and intracellular localization of DYRK1A and DYRK1B was analysed by indirect immunofluorescence staining. Polyclonal antibody: PAb-DYRK1A (ab180910, Abcam); monoclonal antibodies: MAbDYRK1B (ab124960, Abcam); Mab-IE/E used for the detection of HCMV immediate early (IE) IE1p72 and early (E) pUL44 proteins (clones DDG9 and CCH2; Dako); DAPI control staining. Arrows indicate the localization of DYRK1A within the cytoplasmic virion assembly complex (cVAC). (B) MOI-dependent impact of HCMV infection on selected host factors. HFFs were infected with HCMV AD169 at two different MOIs (0.3 or 3; see staining of viral IE1p72, pUL44 and pp28 as an infection control) or remained uninfected as a control (Mock, M). Cells were harvested 24, 48 or 72 hours post-infection and the cell lysates were subjected to SDS–PAGE and Western blot (Wb) analysis using antibodies against the indicated proteins. Polyclonal antibodies: PAb-DYRK1A (#2771, Cell Signaling), PAb-DYRK1B (#2703, Cell Signaling) and PAb-Gli2 (H-300, sc-28674, Santa Cruz Biotechnology). Monoclonal antibodies: MAb-ULK3 (EPR4888, Epitomics), MAb-IE1p72 (63-27), MAb-pp28 (41-18; William Britt, Univ. Alabama, Birmingham, USA), MAb-UL44 (BS510, kindly provided by Bodo Plachter, Univ. Mainz, Germany; Becke et al., 2010), MAb-β-actin (AC-15, Sigma-Aldrich).

Fig. 2. Chemical structures and anti-HCMV activity of DYRK1 inhibitors. (A) SC84227, SC97202 and SC97208 belong to the chemical class of benzohydrofuranes. Chemical formulae and molecular weights (MW) are indicated. (B) Inhibitory activity towards HCMV replication. Compounds were analysed by a GFP-based replication assay using

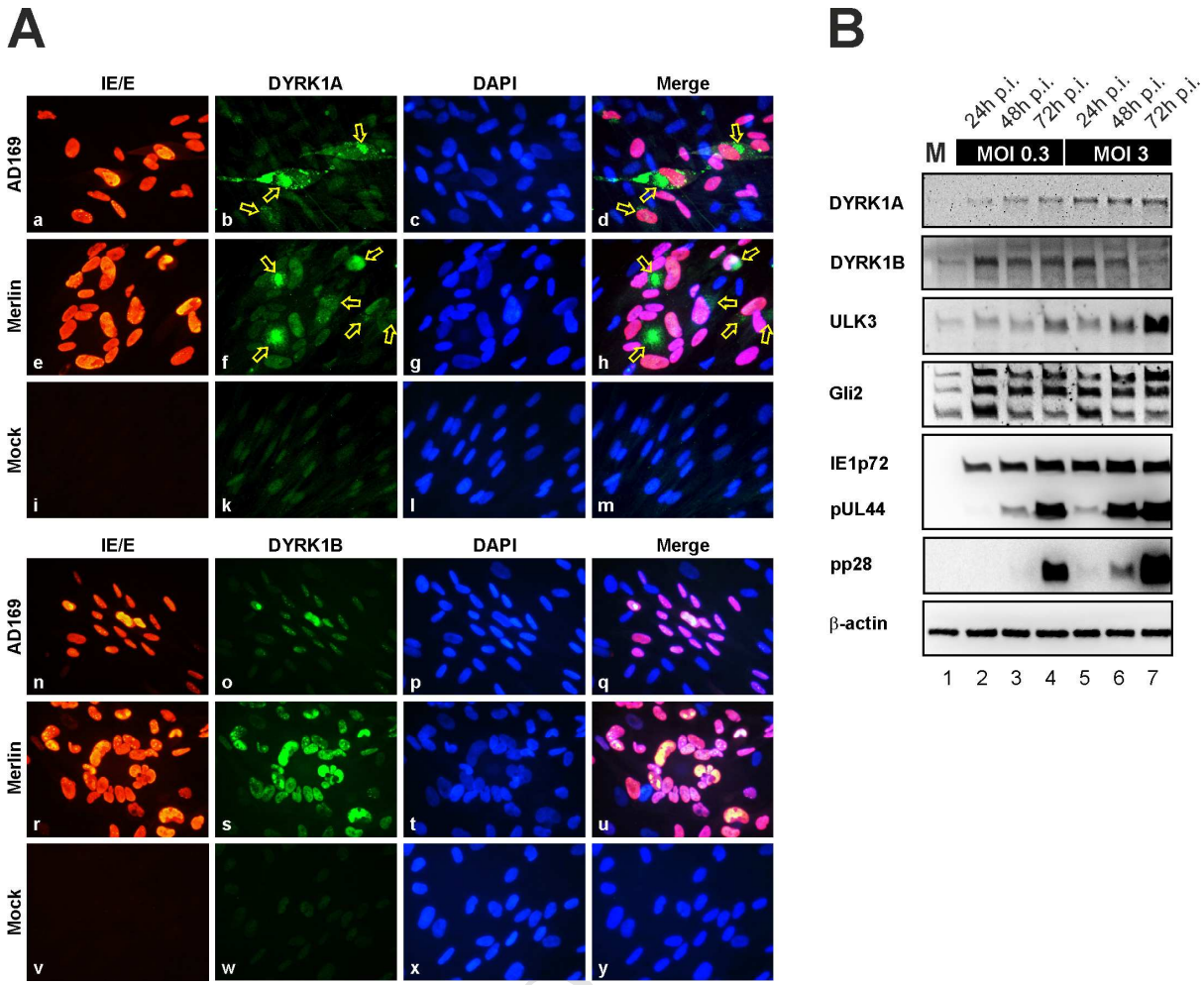
HCMV AD169-GFP for the infection of HFFs (MOI of 0.01; uninfected control, Mock; Marschall et al., 2000). Antiviral compounds were added immediately after infection at the concentrations indicated (reference drug GCV, 20 µM). Cells were lysed seven days post-infection to perform quantitative GFP fluorometry (n = 4, mean ± SD). EC50 values are given above bars.

Fig. 3. Impact of DYRK inhibitors on cell viability or proliferation of primary human fibroblasts. (A) Cytotoxicity was analysed by a lactate dehydrogenase (LDH) release assay (CytoTox 96® NonRadioactive Cytotoxicity Assay, Promega). HFFs (112,000 cells seeded per well of 24-well plates) were treated with DYRK inhibitors AZ-191, Harmine, SC84227, SC97202, SC97208 at the concentrations indicated. Staurosporine (STP, 6 µM) served as a control inducer of cytotoxicity. 24 hours post-treatment, media samples were used for the measurement of LDH release (n = 3). (B) Measurement of cell proliferation in the presence of DYRK inhibitors. Proliferating layers of HFFs (4,500 cells seeded per well of 96-well plates) were incubated with AZ-191, Harmine, SC84227, SC97202, SC97208, or DMSO alone for three days at the concentrations indicated. In the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), the release of NADPH/NADH was indirectly measured by the ability to reduce MTS tetrazolium to formazan, which was quantitated by its absorption at 490 nm. GCV, Ganciclovir, 15 µM, reference compound; FGF-1, fibroblast growth factor 1, 20 ng/ml, used as a proliferation control; STP, staurosporine, 5 µM, used as a control inhibitor.

Fig. 4. Inhibitory effect of DYRK1 inhibitors and transient DYRK1A knockdown, respectively, on HCMV protein production and viral replication. (A) Inhibitory effect of DYRK inhibitors on individual stages of viral protein production. HFFs were infected with HCMV AD169 at a high MOI, or remained uninfected as a control (Mock, M). DMSO or

the following DYRK inhibitors were added immediately after infection: SC83760, 5 µM; SC84227, 5 µM; SC97202, 1.1 µM; SC97208, 15 µM; SC95786, 10 µM; SC99568, 15 µM. Cells were harvested 24, 48 and 72 hours post-infection to perform SDS-PAGE and Western blot analysis using monospecifc antibodies against the indicated proteins. Antibodies are identical to those described for Fig. 1B. (B) Impact of reduced DYRK activity on selected cellular and viral proteins. HFF were infected and treated with DYRK inhibitors or DMSO as described for Fig. 3A. 48 hours post-infection, cells were harvested and subject to Wb analysis using the following antibodies: MAb-ULK3 (EPR4888, Epitomics), PAb-Gli2 (H-300, sc-28674, Santa Cruz Biotechnology) and MAb-Rb (4H1, Cell Signaling), further antibodies used for detection of indicated proteins are identical to those described for Fig. 1B. (C) Effect of a transient siRNA-mediated knockdown of DYRK1A on viral protein expression. HFFs were transfected with a siRNA targeting DYRK1A (siDYRK1A) or with a scrambled siRNA (siControl) as a control siRNA. After siRNA transfection, cells were infected with HCMV AD169-GFP at MOI of 0.5 (Inf.) or not infected (Mock, M.). Cells were harvested 72 hours post-infection to perform SDS-PAGE and Western blot analysis using the following antibodies: PAb-DYRK1A (2771, Cell Signaling), further antibody description see Fig. 1B. (D) Effect of a siRNA-mediated knockdown of DYRK1A on viral replication. HFFs were transfected with a siRNA targeting DYRK1A (siDYRK1A) or with a scrambled siRNA (siControl, J) as a control siRNA. 24 hours post-transfection, HFFs were infected with AD169-GFP at MOI of 0.01 (uninfected control, Mock). Cells were lysed seven days post-infection to perform quantitative GFP fluorometry (n = 4, mean ± SD). Statistical significance, comparing the siDYRK1A panel with siControl or Mock panels, respectively, was obtained by applying unpaired Student's t-test: *P* value ≤ 0.001,***.

Fig. 5. Inhibitory effect of DYRK1 inhibitors and transient DYRK1A knockdown, respectively, on HSV-1 replication and viral protein production. (A) DYRK inhibitors were analysed by a GFP-based replication assay using HSV-1 VP22-GFP for the infection of HFFs (MOI of 0.01, uninfected control, Mock). Antiviral compounds were added immediately post-infection at the concentrations indicated (reference drug ganciclovir, GCV, 20 µM, and cidofovir, CDV, 3 µM). Cells were lysed two days post-infection to perform quantitative GFP fluorometry (n = 4, mean ± SD). (B) Inhibitory effect of AZ-191, Harmine, SC97202, SC97208, SC84227 and CDV on viral protein expression. HFFs were infected at low MOI (~0.5) and kinase inhibitors (5 µl each), reference compound CDV (3 µM) or DMSO alone were added immediately after infection. Cells were harvested 48 hours post-infection to perform SDS-PAGE and Western blot analysis using the following monoclonal antibodies against the indicated proteins: MAb-ICP0 (11060, sc-53070, Santa Cruz Biotechnology), MAb-UL42 (13C9, sc-53331, Santa Cruz Biotechnology), MAb-ICP5 (3B6, sc-56989, Santa Cruz Biotechnology) and MAb-β-actin (AC-15, Sigma-Aldrich). (C) Effect of a siRNA-mediated knockdown of DYRK1A on viral replication. HFFs were transfected with a siRNA targeting DYRK1A (siDYRK1A) or with a scrambled siRNA (siControl, G) as a control siRNA. 24 hours post-transfection, HFFs were infected with HSV-1 VP22-GFP at very low MOI (uninfected control, Mock). Cells were lysed three days post-infection to perform quantitative GFP fluorometry (n = 4, mean ± SD). Statistical significance, comparing the siDYRK1A panel with siControl or Mock panels, respectively, was obtained by applying unpaired Student's t-test: *P* value ≤ 0.01,**; ≤ 0.001,***.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IE/E
DYRK1A
DAPI
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AD169
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Mock
a
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IE/E
DYRK1B
DAPI
Merge
AD169
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24h p.i.
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72h p.i.
24h p.i.
48h p.i.
72h p.i.
M
MOI 0.3
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ULK3
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pUL44
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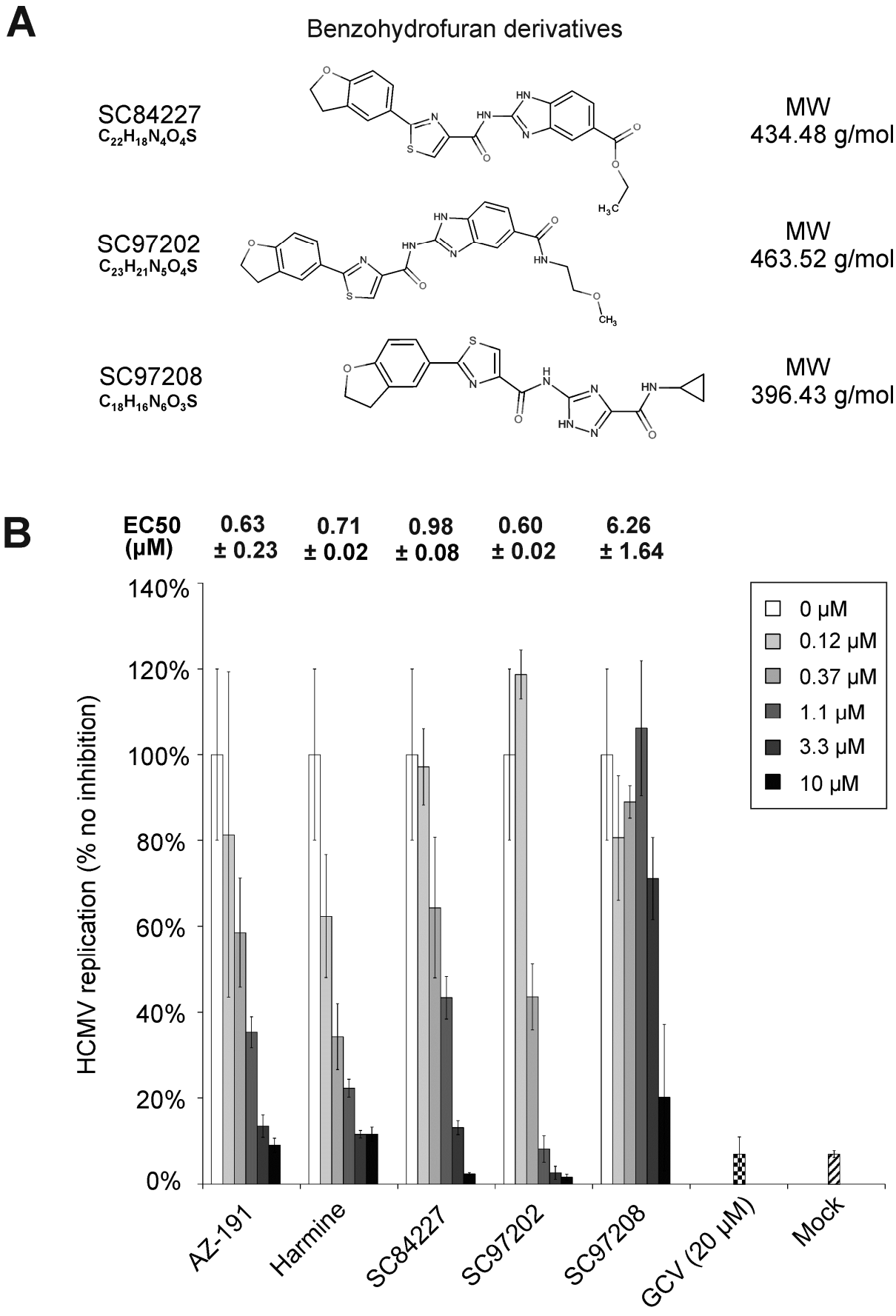
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SC97202
C23H21N5O4S
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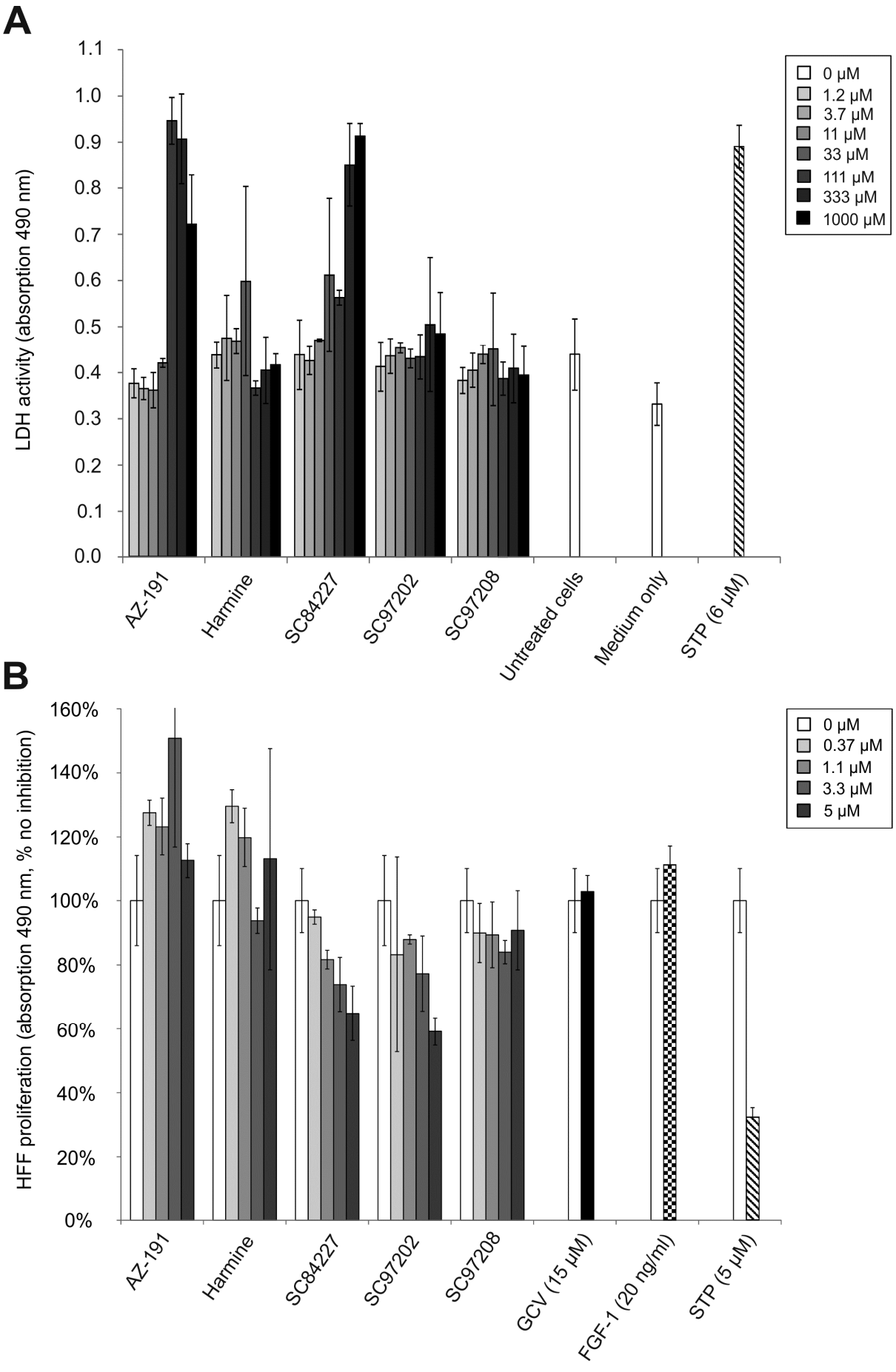
463.52 g/mol
SC97208
C18H16N6O3S
MW
396.43 g/mol
B
EC50 (μM)
0.63 ± 0.23
0.71 ± 0.02
0.98 ± 0.08
0.60 ± 0.02
6.26 ± 1.64
HCMV replication (% no inhibition)
140%
120%
100%
80%
60%
40%
20%
0%
0 μM
0.12 μM
0.37 μM
1.1 μM
3.3 μM
10 μM
AZ-191
Harmine
SC84227
SC97202
SC97208
GCV (20 μM)
Mock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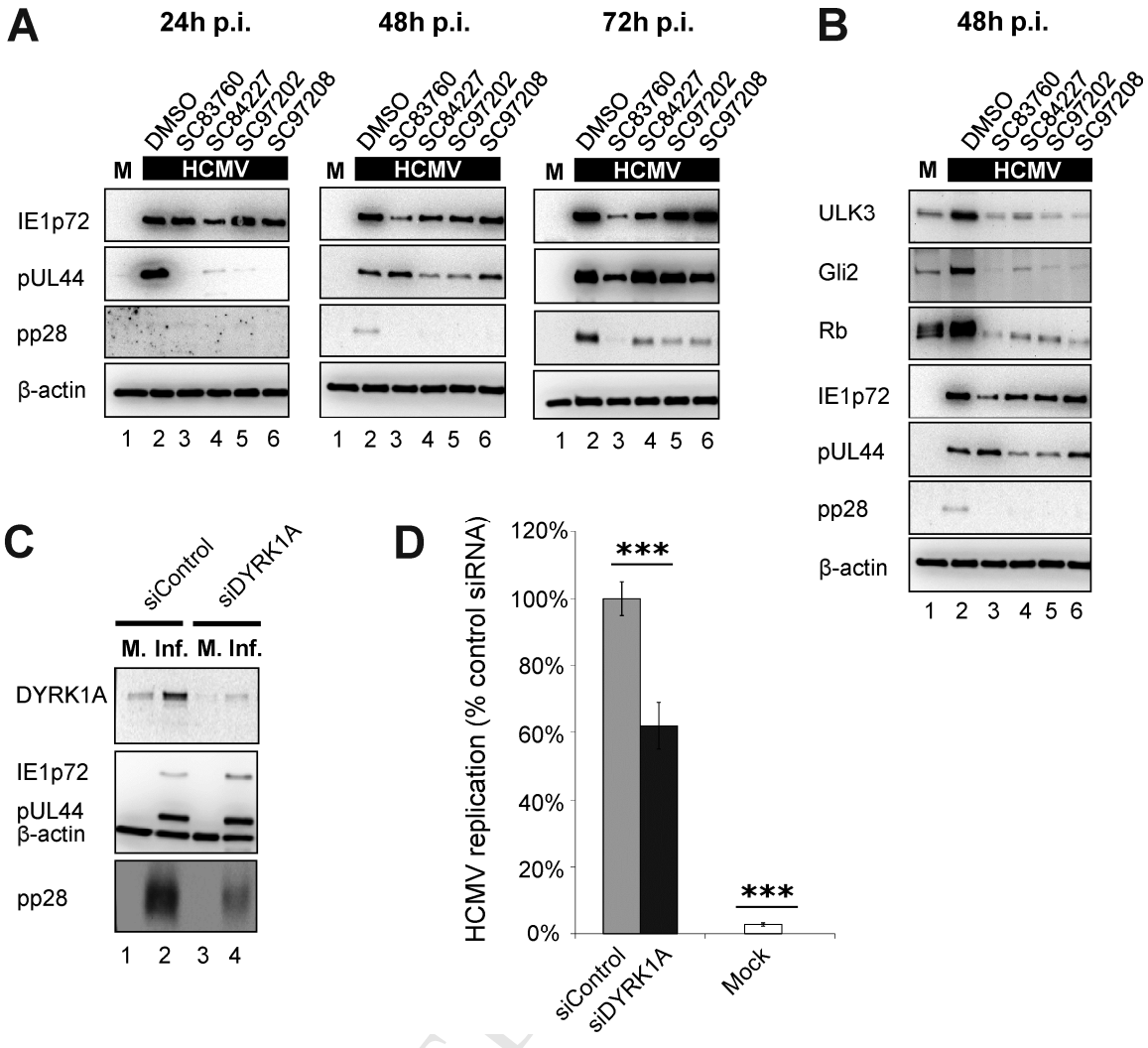
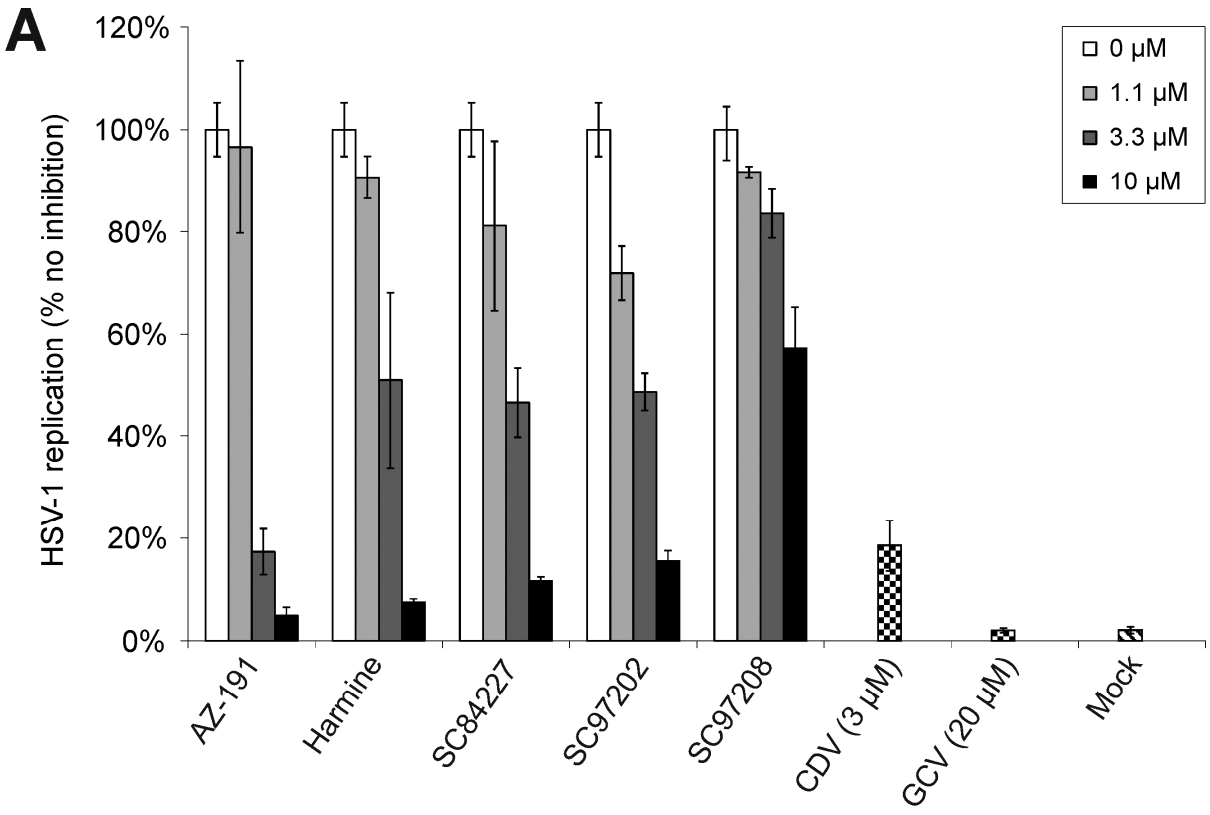
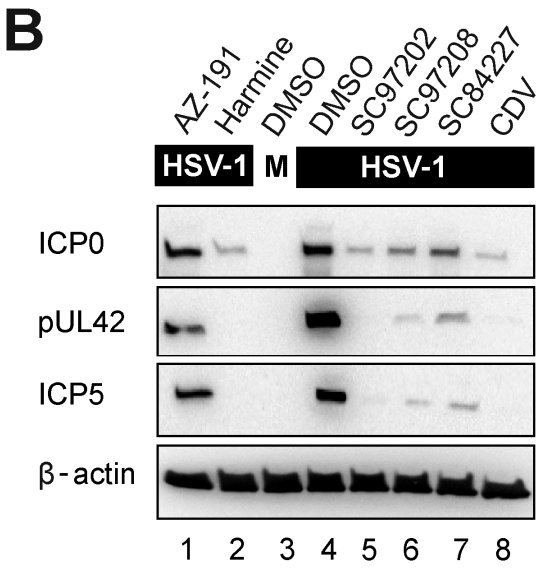
LDH activity (absorption 490 nm)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 µM
1.2 µM
3.7 µM
11 µM
33 µM
111 µM
333 µM
1000 µM
AZ-191
Harmine
SC84227
SC97202
SC97208
Untreated cells
Medium only
STP (6 µM)
B
HFF proliferation (absorption 490 nm, % no inhibition)
160%
140%
120%
100%
80%
60%
40%
20%
0%
0 µM
0.37 µM
1.1 µM
3.3 µM
5 µM
AZ-191
Harmine
SC84227
SC97202
SC97208
GCV (15 µM)
FGF-1 (20 ng/ml)
STP (5 µM)




A
120%
100%
80%
60%
40%
20%
0%
HSV-1 replication (% no inhibition)
0 µM
1.1 µM
3.3 µM
10 µM
AZ-191
Harmine
SC84227
SC97202
SC97208
CDV (3 µM)
GCV (20 µM)
Mock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AZ-191
Harmine
DMSO
DMSO
SC97202
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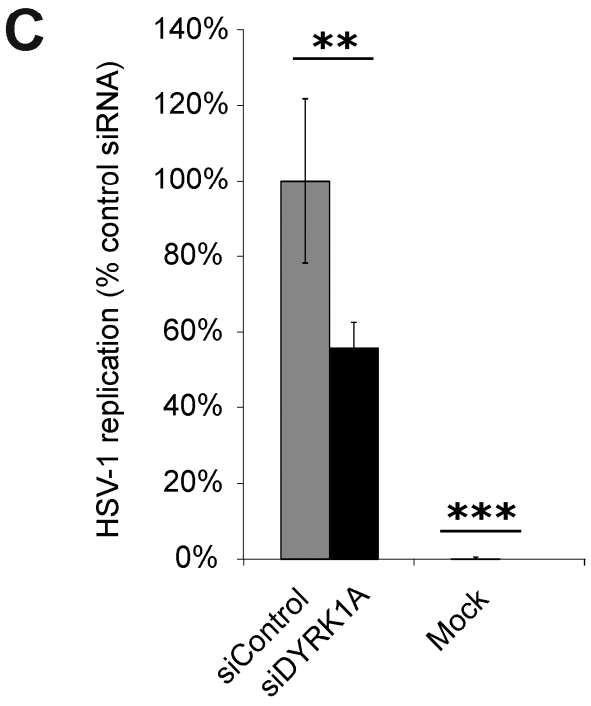
C
140%
120%
100%
80%
60%
40%
20%
0%
HSV-1 replication (% control siRNA)
**
***
siControl
siDYRK1A
Mock

Highlights

The main findings of the study are:

(i) First evidence for a critical role of dual-specificity tyrosine phosphorylation-regulated kinases during viral replication

(ii) Novel DYRK inhibitors (benzohydrofurane derivatives) exert strong anti-herpesviral activity

(iii) Knockdown of DYRK1A impairs efficient replication of HCMV and HSV-1 in fibroblasts

(iv) A block of viral replication occurs at the early-late stage of HCMV gene expression

(v) DYRK kinases may represent novel targets for host cell-directed drugs