



Regulation of influenza A virus mRNA splicing by CLK1

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ABSTRACT

Influenza A virus carries eight negative single-stranded RNAs and uses spliced mRNAs to increase the number of proteins produced from them. Several genome-wide screens for essential host factors for influenza A virus replication revealed a necessity for splicing and splicing-related factors, including Cdc-like kinase 1 (CLK1). This CLK family kinase plays a role in alternative splicing regulation through phosphorylation of serine-arginine rich (SR) proteins. To examine the influence that modulation of splicing regulation has on influenza infection, we analyzed the effect of CLK1 knockdown and inhibition. CLK1 knockdown in A549 cells reduced influenza A/WSN/33 virus replication and increased the level of splicing of segment 7, which encodes the viral M1 and M2 proteins. *CLK1* $-/-$ mice infected with influenza A/England/195/2009 (H1N1pdm09) virus supported lower levels of virus replication than wild-type mice. Screening of newly developed CLK inhibitors revealed several compounds that have an effect on the level of splicing of influenza A gene segment M in different models and decrease influenza A/WSN/33 virus replication in A549 cells. The promising inhibitor KH-CB19, an indole-based enaminonitrile with unique binding mode for CLK1, and its even more selective analogue NIH39 showed high specificity towards CLK1 and had a similar effect on influenza mRNA splicing regulation. Taken together, our findings indicate that targeting host factors that regulate splicing of influenza mRNAs may represent a novel therapeutic approach.

1. Introduction

The process of alternative splicing diversifies gene expression, with 75% of human genes having at least two alternatively spliced isoforms. Splicing is highly regulated via recognition of sites by different proteins and synergistic effects of multiple weak binary-interactions (Schellenberg et al., 2008; Wahl et al., 2009). Several viruses also

employ alternative splicing to code for diverse proteins from their small genomes (Fukuhara et al., 2006; Hryckiewicz et al., 2011; Johansson and Schwartz, 2013; Ortín, 1998). Influenza A virus (IAV) makes use of this process to generate several of its proteins (Lamb and Lai, 1980; Lamb et al., 1981; Wise et al., 2012). It belongs to the family of *Orthomyxoviridae* and carries a genome consisting of eight negative single-stranded viral RNAs (vRNAs). According to current knowledge, the HA,

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NP (nucleoprotein) and NA segments each encode only one viral protein, while the PB1, PB2, PA, M and NS segments give rise to two or more mRNAs via alternative reading frame, differential initiation at AUG codons, ribosomal frameshifting or splicing (Chen et al., 2001; Jagger et al., 2012; Lamb and Lai, 1980; Lamb et al., 1981; Wise et al., 2009, 2012). The unspliced M mRNA encodes for M1 matrix protein while the spliced M2 mRNA encodes the M2 ion-channel protein (Inglis and Brown, 1981; Lamb et al., 1981). A third splice variant, M3 mRNA, is not required for replication of influenza virus *in vitro* (Jackson and Lamb, 2008) and it remains unclear if any protein is expressed from it. The M4 mRNA uses the 5' splice site (ss) at position 146 and exists only in some strains, such as A/WSN/33 (Shih et al., 1998). Mutation at the 5'ss of M4 mRNA impairs M mRNA splicing and virus growth via an unclear mechanism (Chiang et al., 2008). An M2-related protein named M42 is expressed from M4 mRNA utilizing an alternative start codon and is hypothesized to replace the function of M2 (Wise et al., 2012). The NS segment also undergoes splicing, with the unspliced mRNA encoding the non-structural protein NS1, while the spliced mRNA encodes the nuclear export protein NS2/NEP (Lamb and Lai, 1980). Recently, the PB2 segment has also been shown to produce a spliced mRNA that encodes PB2-S1, which, like the unspliced PB2, inhibits RIG-I-dependent IFN signaling (Yamayoshi et al., 2015).

Viral mRNA splicing utilizes the cellular splicing machinery and is tightly regulated to maintain adequate expression of the unspliced mRNA. Several mechanisms have so far been found for this: The cellular splicing factor SF2/ASF binds to a purine-rich splicing enhancer sequence at the 3' exon of M1 mRNA to control production of M2 mRNA and protein synthesis (Shih and Krug, 1996). NS1 protein regulates splicing of its own mRNA (Garaigorta and Ortin, 2007), as well as splicing and export of M mRNA (Robb and Fodor, 2012). The cellular protein NS1-BP, which binds influenza NS1 protein, also promotes pre-mRNA splicing (Wolff et al., 1998). In addition, the rate of NS1 mRNA splicing is controlled by cis-acting sequences of the mRNA itself as well as nucleocytoplasmic transport of the unspliced mRNA (Alonso-Caplen and Krug, 1991). A recent report elucidated a similar cis-acting regulatory element in NS mRNA that is an exonic splice enhancer, which utilizes the NS1 protein itself and the host factor SF2 (Huang et al., 2017).

Previous genome-wide RNAi screens have identified CLK1 as a host cell factor required for influenza A virus replication (Karlás et al., 2010; König et al., 2010). CLK1 belongs to the family of human protein kinases, which has high homology to the yeast *cdc2/CDC28* kinases (Johnson and Smith, 1991). There are four isoforms of CLK in mammals, CLK1, 2, 3 and 4 (Hanes et al., 1994; Nayler et al., 1997). CLK1 phosphorylates the C-terminus of SR proteins, a family of splicing factors, to regulate their subcellular distribution and activity (Colwill et al., 1996; Duncan et al., 1997; Ngo et al., 2005). Overexpression of CLK1 is known to promote use of the distal 5'ss of the adenoviral E1A gene (Duncan et al., 1997). Similarly, processing of HIV mRNA is affected by overexpression of CLK isoforms (Wong et al., 2011), while inhibition of the SR protein-specific kinases SRPK1 and 2 (Ngo et al., 2008) leads to reduced HIV production (Fukuhara et al., 2006). Different inhibitors of CLK1 can reduce HIV replication *in vitro* via effects on mRNA processing, with the notable caveat that specificity for different CLK isoforms appears to have a large influence on the effectiveness (Wong et al., 2011, 2013). In the same way, we have previously found that the CLK inhibitor TG003 alters IAV M mRNA splicing and M protein expression *in vitro* (Karlás et al., 2010).

Here, we describe that inhibiting CLKs in host cells with small molecule inhibitors impaired IAV replication. The degree to which splicing was altered and the extent to which replication was inhibited was dependent on the specificity of the CLK inhibitor. Assessment of the downstream targets of CLK family kinases identified SRSF3 as the main SR protein which affects mRNA splicing and IAV replication. We conclude that proper viral mRNA splicing is required for efficient replication of IAV.

2. Materials and methods

2.1. Cell lines and viruses

Human lung epithelial cells (A549, CCL-185, ATCC-LGC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS, Biochrome), 4 mM L-glutamine and 4 mM sodium pyruvate at 37 °C and 5% CO₂. Madin–Darby canine kidney cells (MDCK, CCL-34, ATCC-LGC) were grown in DMEM supplemented with 10% fetal calf serum, 4 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37 °C and 5% CO₂. Influenza virus A/WSN/33 and A/England/195/2009 virus was propagated in allantoic fluid of eleven-day old embryonated chicken eggs as previously described (Szretter et al., 2006). The recombinant wild-type virus A/Victoria/3/75 (H3N2) was generated by reverse genetics, as described previously (Elleman and Barclay, 2004; Neumann et al., 1999; Roberts et al., 2011). The titer of virus stocks was determined by plaque assay on MDCK cells using agar overlay medium (Szretter et al., 2006).

2.2. Chemical inhibitor treatment in eukaryotic cells

Chemical inhibitor was diluted to the specified concentration in culture medium, added to A549 cells and incubated at 37 °C with 5% CO₂ for 2 h. DMSO diluted in culture medium at the same volume as the inhibitor served as control. After 2 h, cells were infected with influenza virus A/WSN/33 diluted in infection buffer at the specified MOI and incubated at room temperature for 1 h. Virus was then removed, replaced with infection medium containing the chemical inhibitor or DMSO at the specified concentration and incubated at 37 °C with 5% CO₂. TG003 was purchased from Sigma.

2.3. CRISPR/Cas9-mediated CLK1 knockout cells

A549 cells lacking a functional CLK1 gene were generated as described (Karlás et al., 2016). Briefly, Cas9-expressing A549 cells were generated by transducing with lentiviruses based on the plasmid lentiCas9-Blast (Addgene number 52962), followed by selection with blasticidin. Cells were then transduced with lentivirus derived from the plasmid lentiGuide-Puro44 (Addgene number 52963), which leads to the expression of CLK1-specific gRNAs.

2.4. SC35 subnuclear distribution detection upon CLK overexpression and inhibitor treatment

A549 cells were transfected with plasmid expressing GFP-tagged mCLK1, mCLK2, mCLK3 and mCLK4 using Lipofectamine LTX (Invitrogen) according to manufacturer's instruction. The plasmids expressing GFP-tagged mCLKs were a kind gift from Alan Cochrane (University of Toronto, Canada). At 16 h post-transfection, cells were treated with 50 μM TG003, 12.5 μM NIH39 or 50 μM kH-CB19 for 6 h, washed once with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 1% Triton X-100, followed by blocking in 1xPBS containing 1% BSA/0.5% gelatine. Subnuclear distribution of SC35 was detected by labelling with mouse anti-SC35 antibody (BD Science) followed by Cy3-labelled anti-mouse IgG (Jackson Immuno Research Lab) and Hoechst, as described above. Images were captured using a Leica DMR microscope.

2.5. Luciferase splicing assay

To quantify influenza segment M splicing, a cell-based influenza-driven splicing assay that monitors expression of the three mRNAs produced from the M gene (M1, M2 and M3) was used (Moncorgé O. and Barclay W., in preparation). Briefly, plasmids that generate *in situ* viral-like RNAs that can be amplified and expressed by influenza virus polymerase are transfected in 293T cells 24 h prior to viral infection.

The viral-like RNAs are based on segment M sequence (A/England/195/2009, H1N1pdm09) and retain all the documented control regions potentially involved in splicing (Lamb and Lai, 1982; Moss et al., 2011, 2012; Shih and Krug, 1996). Instead of encoding the authentic virus products, the reporter RNAs encode luciferase proteins in frame with the M1 (unspliced), M2 (spliced) or M3 (spliced) mRNA open reading frames. This allows quantification of expression of proteins that arise from the various alternative splicing routes by a dual luciferase assay on lysates of infected cells. 24 h post-infection (MOI 1), cells are lysed using 5x passive lysis buffer (Promega) and luciferase activity is measured.

2.6. LabChip kinase screening

Enzymatic kinase screening was carried out using the Caliper mobility shift assay, which is based on the difference in capillary electrophoresis mobility of a fluorescently tagged peptide as a result of the addition of a phosphate moiety by the studied kinase. The consensus peptide for CLK1 (AFRREWSPGKEAKK) was solubilized at 10 mM in DMSO and used as a substrate at a final concentration of 1 μ M and the CLK1 enzyme at 0.945 ng in each reaction in 30 μ l volumes. 0.3 μ l each of compound solution (1 mM in DMSO) was added to 10 μ l assay buffer (50 mM HEPES [pH 7.5], 80 mM NaCl, 600 μ M MgCl₂, 1 mM DTT, 0.002% Tween 20). After addition of 10 μ l CLK1 in assay buffer, the reaction was started by adding 10 μ l of substrate solution with consensus peptide and ATP at concentrations set to the KM for CLK1 at 19.2 μ M. After incubation for 120 min at 20 °C 50 μ l stop solution was added (100 mM HEPES [pH 7.5], 5% DMSO, 0.1% coating reagent [Caliper Lifescience] 10 mM EDTA [pH 8.0], 0.015% BRIJ35) and reactions analyzed in a LC3000 reader (Caliper Life Sciences) using the following settings: DS -600, US -2300, base pressure - 0.5, screen pressure - 1.8, plate cycles 1, end of plate delay 60, final delay 30, sample time 0.2, post sample buffer time 35, dye time 0.2, post-dye buffer time 40. Validation of dosage-dependent inhibitory activity was carried out by IC₅₀ determinations in serial 1:1 dilutions from 50 μ M to 50 nM in ten steps and in duplicate. For automated data analysis, the LabChip files are converted to a standardized output format (in-house developed software) and fed into statistical analysis by the open source software R and KNIME to generate exact curve fittings and IC₅₀ calculations.

2.7. Animal experiments

Animals were housed and bred under pathogen-free conditions, in a BSL 2 laboratory according to the German Animal Protection Law (Tierschutzgesetz TierSchG). Animal testing was approved by the local authorities (Landesamt für Gesundheit und Soziales Berlin) under license G0206/12. C57BL/6/J and CLK1^{-/-} mice were obtained from Charles River and the European Conditional Mouse Mutagenesis Program (EUCOMM) Consortium, respectively, and further bred in-house. C57BL/6 mice aged 11–16 weeks and weighing 20.9–25.6 g and CLK1^{-/-} mice aged 7 weeks and weighing 20.4–24.0 g were intranasally infected with 9×10^4 PFU of influenza A/England/195/2009 virus in 30 μ l infection buffer. Two days later, lungs of infected animals were isolated and homogenized in PBS, followed by centrifugation at $800 \times g$ for 8 min at 4 °C. The amount of infectious virus was then quantified using plaque assay.

3. Results

3.1. CLK1 knockdown reduces influenza A virus replication and regulates the splicing of viral mRNA

CLK1 was identified as an essential host factor for influenza A virus infection in two previous screens (Karlas et al., 2010; König et al., 2010). To verify the result, we performed knockdown experiments in

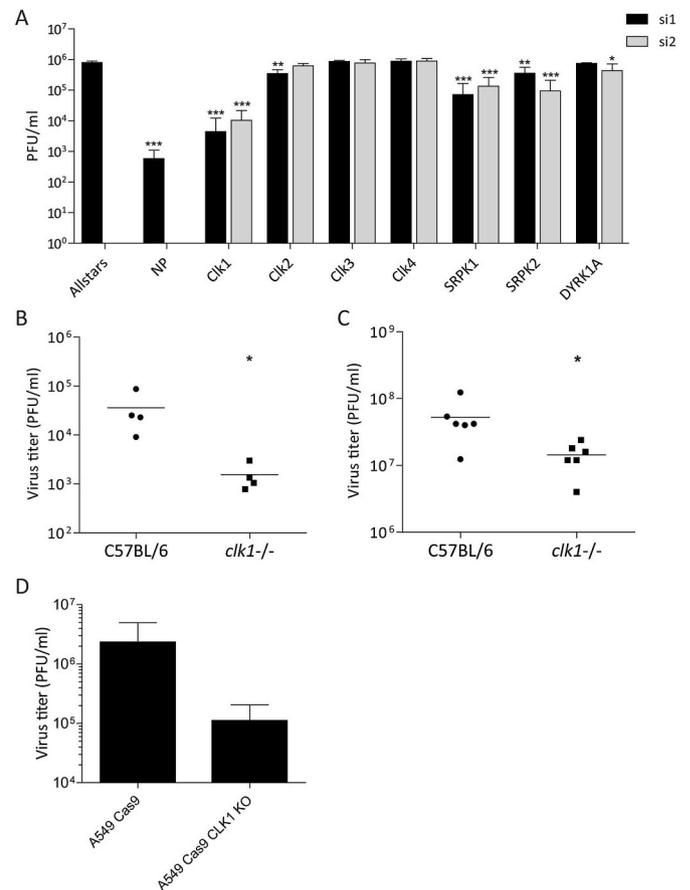


Fig. 1. CLK1 is an essential host factor for influenza A virus replication. (A) Replication assay was carried out by infection of A549 cells 48 h post siRNA transfection with influenza A/WSN/33 at MOI of 0.01 for 36 h. The titer of infectious viral particles in the supernatant was measured using indirect immunofluorescence staining (shown are mean values of three independent experiments \pm SEM). *p-value < 0.05, **p-value < 0.01, ***p-value < 0.0001, one-way ANOVA. (B) Cells isolated from the lungs of either C57BL/6 wild-type or CLK1^{-/-} mice were infected with influenza A/WSN/33 at MOI 0.01 for 72 h. Production of infectious viral progeny was measured using indirect immunofluorescence staining. *p-value < 0.05, two-tailed unpaired *t*-test. (C) C57BL/6 wild-type or CLK1^{-/-} mice (*n* = 6) were intranasally infected with 9×10^4 PFU of influenza A/England/195/2009 (H1N1pdm09). At 48 h after infection, infectious viral particles within the lungs were quantified using plaque assay. *p-value < 0.05, two-tailed unpaired *t*-test. (D) Validation of CLK1 as relevant cellular factor. Amount of virus produced in A549 cells depleted for CLK1 by CRISPR/Cas9 and infected with A/WSN/33 at MOI 0.001 for 24 h. Virus titer was quantified by plaque assay (shown are mean values of three independent experiments \pm SEM).

A549 cells to silence kinases that regulate splicing, including the CLK and SRPK family kinases and the dual specificity tyrosine phosphorylation-regulated kinase 1 (DYRK1A), followed by infection with influenza A/WSN/33 at MOI 0.01 for 36 h. The infectious viral particles produced were quantified by reinfection of MDCK cells, followed by indirect immunofluorescence staining. Among the CLK family kinases, only knockdown of CLK1 significantly reduced the production of virus progeny with both siRNAs (Fig. 1A), compared to the negative siRNA control Allstars. There was also a significant reduction in virus replication after knockdown of SRPK1 and SRPK2 with both siRNAs, albeit 10-fold less than that seen with CLK1 (Fig. 1A). Knockdown did not significantly affect host cell viability at 48 h post-transfection (Fig. S1A) and most of the siRNAs used for the knockdowns reduced gene expression efficiently, as quantified by qPCR (Fig. S1B). Due to the strong similarity between the different CLK isoforms, especially between CLK1 and CLK4, the quantification of knockdown efficacies on protein level is

not feasible.

To further verify the importance of CLK1 in influenza A virus replication, we isolated cells from the lungs of *CLK1*^{-/-} and C57BL/6 mice. Equal numbers of isolated cells were infected with influenza A/WSN/33 at MOI 0.01 for 72 h. Viral replication was significantly reduced when compared to control cells isolated from wild-type C57BL/6 mice (Fig. 1B), despite the fact that even in the *CLK1*^{-/-} cells a slight expression of CLK1 was detectable (Fig. S1C). To confirm this effect *in vivo*, intranasal infection of C57BL/6 and *CLK1*^{-/-} mice was performed with influenza A/England/195/2009 for two days. Determination of virus titer in the lungs harvested at 2 days post-infection showed that the number of infectious viral particles obtained from *CLK1*^{-/-} mice was significantly reduced (Fig. 1C). Similarly, A549 cells lacking CLK1 expression after CRISPR/cas9-mediated knockout replicated influenza viruses less efficiently than controls (Fig. 1D).

As CLK1 is known to regulate mRNA splicing, we next investigated whether knockdown has any effect on splicing of influenza mRNAs. qRT-PCR quantification of spliced and unspliced viral mRNAs upon infection with A/WSN/33 at MOI 4 for 5 h showed an increased ratio of spliced to unspliced M mRNA in CLK1 knockdown A549 cells, but no significant effect on NS mRNA splicing was observed compared to the negative control Allstars (Fig. 2A). Western blot analysis also showed that expression of the unspliced viral M1 and NS1 proteins was reduced upon CLK1 knockdown, but little or no effect on the level of the spliced M2 protein was observed (Fig. 2B).

3.2. Screening of CLK inhibitors identifies new compounds that inhibit influenza virus replication

We previously observed that cells treated with the CLK inhibitor TG003 showed reduced influenza A/WSN/33 virus replication (Karlas et al., 2010). This prompted us to screen 60 new compounds (structurally related to KH-CB19, previously published as a CLK1 inhibitor (Fedorov et al., 2011)) targeting CLK family kinases in an IAV replication assay. Several of these compounds inhibited influenza replication to a greater extent than the commercially available TG003 (Fig. 3A), without significantly affecting cell viability at 48 h post-treatment (Fig. S2). Two of these were selected for further experiments - NIH39 and KH-CB19, which had IC50 values of 6.6 μ M and 13.6 μ M for antiviral activity, respectively (Fig. 3B and C). Treatment of A549 cells with NIH39 at 6.25 μ M or 12.5 μ M upon infection with A/WSN/33 at MOI 4 for 5 h resulted in an increase of the spliced to unspliced viral M mRNA ratio (Fig. 4A, Fig. S3A). Unexpectedly, however, qRT-PCR

analysis showed that the viral NS mRNA splicing ratio was significantly reduced when cells were treated with 12.5 μ M NIH39 (Fig. 4A, Fig. S3B). Viral M1, M2 and NS1 protein levels were all found to be markedly reduced when analyzed by Western blot (Fig. 4B, Fig. S3E). In contrast to the effects observed with NIH39 treatment, A549 cells treated with KH-CB19 at 50 μ M or 100 μ M did not show any effect on the spliced to unspliced ratio of viral M and NS mRNAs (Fig. 4C, Figs. S3C–D), although the levels of all three viral proteins were also reduced (Fig. 4D, Fig. S3F).

It has been reported previously that overexpression of CLK family kinases alters the subnuclear distribution of the SR protein by altering its phosphorylation state (Wong et al., 2011, 2013). The observed differential effects of the various CLK inhibitors on influenza mRNA splicing raised the question whether they affected the four CLK isoforms differently. Therefore, we next tested the specificity of the inhibitors by analysing the nuclear speckle pattern of SC35 (SRSF2) labelling upon mClk overexpression (Wong et al., 2011). In accordance with the previous reports, CLK overexpression altered the subnuclear distribution of SC35 from a speckled to a diffuse pattern (Fig. 5A). Treatment of cells with TG003 inhibited nuclear speckle disruption by CLK1, 2 and 4 (Fig. 5B). This confirms reports that TG003 inhibits CLK1, CLK4 and to a lesser extent CLK2 (Muraki et al., 2004; Wong et al., 2011). NIH39 treatment blocked the disruption of nuclear speckles by CLK1 and 4 (Fig. 5C, Fig. S6), while KH-CB19 only inhibited the disruption by CLK4 (Fig. 5D, Fig. S6).

3.3. Identification of the essential SR protein in influenza A virus replication and its effect on viral mRNA splicing

To identify the downstream targets of CLK1 in influenza infection, knockdown of SR proteins was performed in A549 cells, followed by influenza virus replication assay and cell viability analysis. Knockdown of SRSF3 with each of two siRNAs significantly impaired influenza virus replication, while the other SRSFs had a less pronounced effect or showed strong reduction only with one siRNA (Fig. 6A). Host cell viability at 48 h after siRNA transfection was not significantly impaired upon knockdown of any SRSF proteins (Fig. S4A) and all siRNAs achieved efficient knockdown (Fig. S4B).

Next, we further analyzed viral mRNA splicing upon SRSF3 knockdown (Fig. S4C). Quantification of spliced and unspliced viral mRNAs was performed in A549 cells after knockdown, followed by infection with influenza A/WSN/33 at MOI 4 for 5 h. The result shows that in the absence of SRSF3 there is an increase in the viral M and NS mRNA splicing ratios (Fig. 6B, Figs. S4D–E). Remarkably, in-depth analysis of viral M and NS mRNA sequences using ESEfinder and SFmap showed different binding sites for several SR proteins between the M and NS mRNA including binding sites for SRSF3 (Fig. S7). On the protein level, SRSF3 knockdown slightly reduced the levels of viral M1 and NS1 proteins, as we observed with CLK1, and slightly increased the level of the spliced M2 protein (Fig. 6C, Fig. S4F). Based on these results, SRSF3 seems to be the main actor influencing the levels of spliced viral RNAs upon removal or inhibition of CLK1.

3.4. Improved CLK1 inhibitors

The CLK1 inhibitors that performed best in our assays, NIH39 and KH-CB19, had antiviral IC50 values of 6.6 μ M and 13.6 μ M, respectively. In the search for a therapeutic intervention based on splicing inhibition, we decided to further improve the compound efficacy. We started a rational drug development program supported by (i) virtual screening of inhibitor libraries, (ii) enzymatic screening using luciferase- and LabChip-based assay systems and (iii) screening of selected inhibitors regarding antiviral efficacy and cell viability.

We designed a library of 580 compounds by docking based on the molecular structure of the hits and by docking of putative inhibitors into the active site of the published CLK1 crystal structure (PDB-code

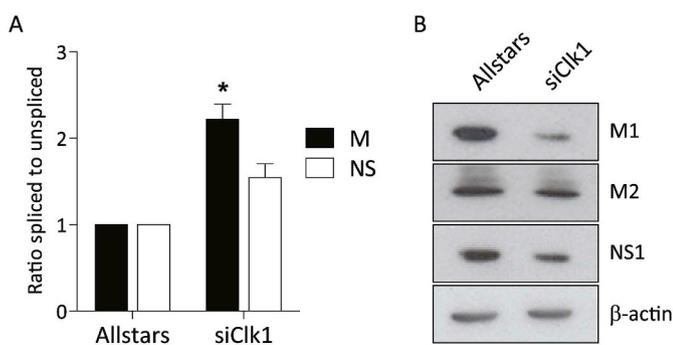


Fig. 2. Effect of CLK1 knockdown on influenza mRNA and protein levels. A549 cells were infected with influenza A/WSN/33 at MOI 4 for 5 h, at 48 h after transfection with siRNA targeting CLK1 or non-targeting Allstars control siRNA. (A) Ratio of spliced to unspliced mRNA upon CLK1 knockdown was calculated by dividing the relative spliced mRNA level by the relative unspliced mRNA level. Results represent mean \pm SEM of three independent experiments; *p-value < 0.05, two-tailed column statistics compared to the non-targeting Allstars control. (B) The influenza M1, M2 and NS1 protein levels upon CLK1 knockdown were examined using Western Blot. Depicted immunoblots are representative of three independent experiments.

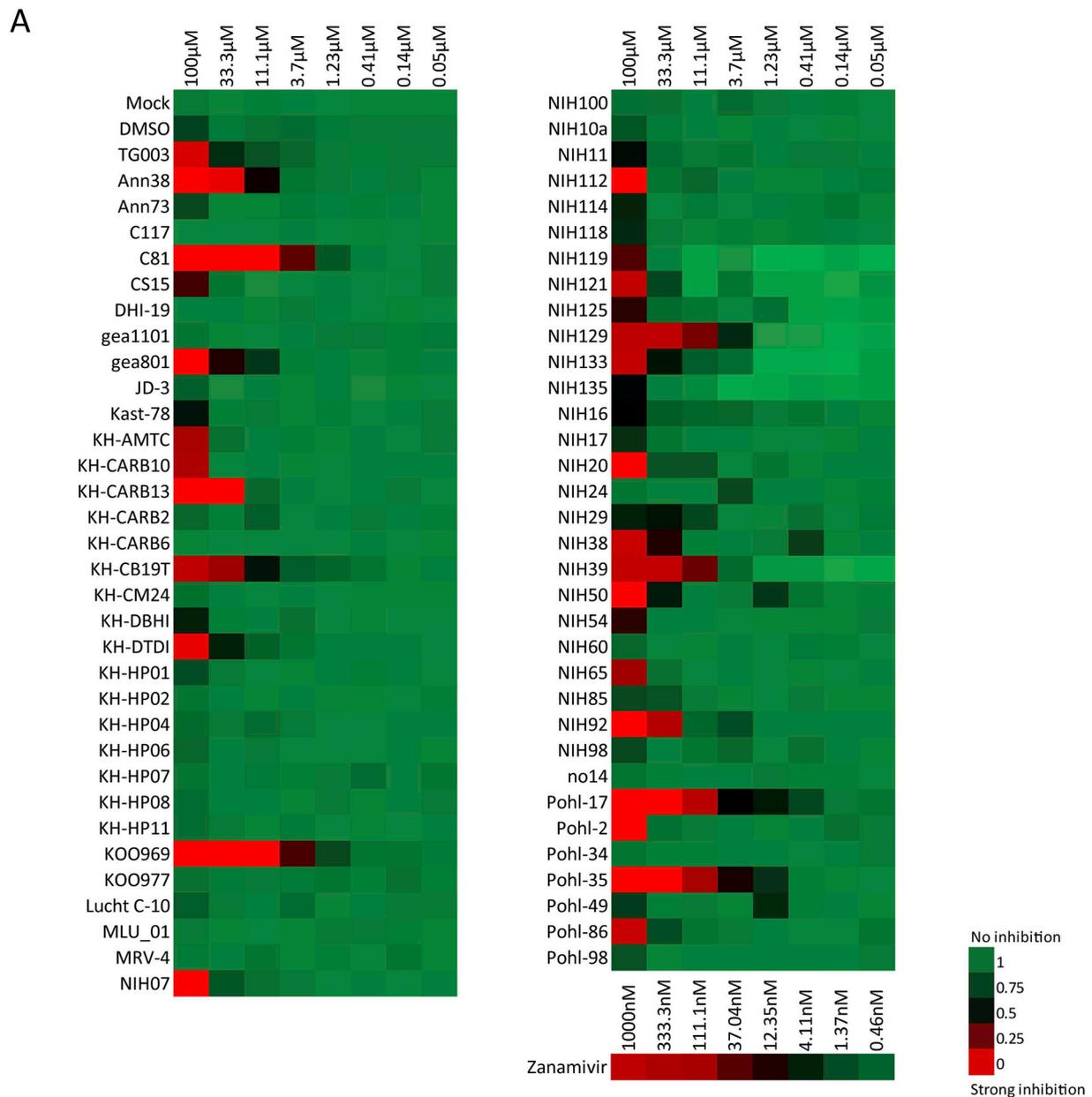


Fig. 3. Screening of CLK inhibitors affecting influenza virus replication (A) A549 cells were treated with eight serial dilutions of the inhibitor dissolved in DMSO. Two h later, cells were infected with A/WSN/33 at MOI of 0.0095 for 40 h. Infectious viral particles produced in the presence of the inhibitor were quantified using indirect immunofluorescence staining and calculated as normalized percentage of infection from three independent experiments. (B,C) IC₅₀ curve of NIH39 (B) and KH-CB19 (C) activity on influenza virus replication, as well as the effect of inhibitor treatment on A549 cell viability (red lines). Data represent mean \pm SEM of at least three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

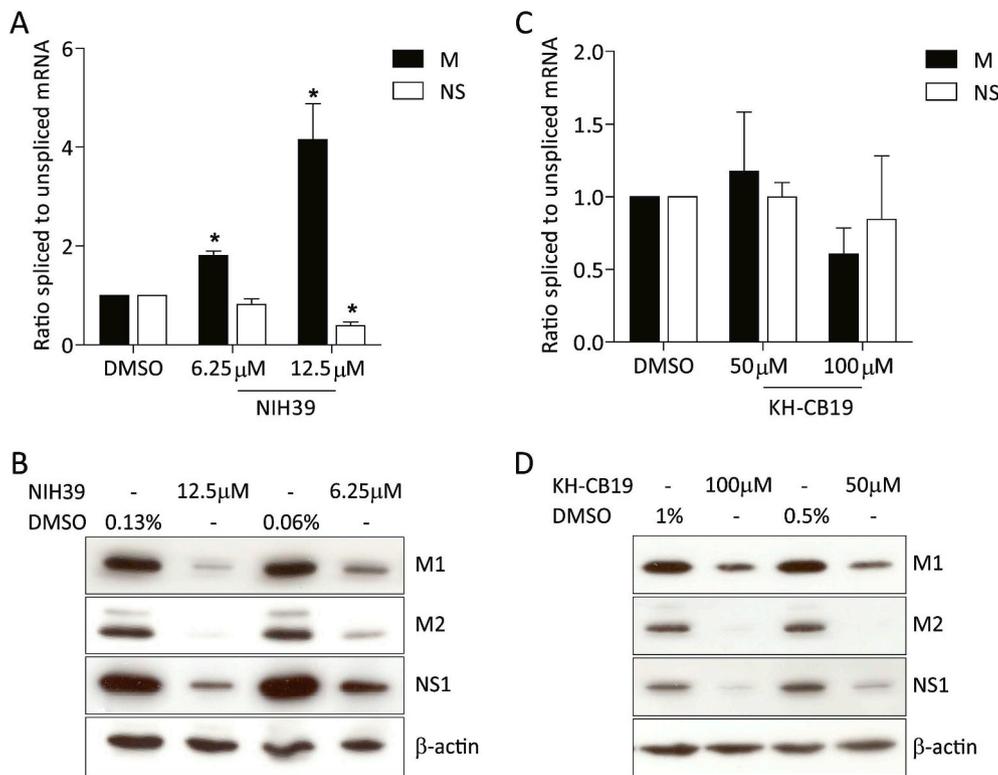


Fig. 4. NIH39 and KH-CB19 treatment lead to changes in influenza mRNA splicing and protein expression. (A,C) Ratio of viral spliced to unspliced mRNA upon treatment with NIH39 (A) or KH-CB-19 (B). A549 cells were pre-treated with inhibitors or dimethylsulphoxide (DMSO) for 2 h and subsequently infected with influenza A/WSN/33 at MOI 4 for 5 h. Results represent mean \pm SEM of at least three independent experiments; *p-value < 0.05, two-tailed column statistics compared to the DMSO control. (B,D) Influenza M and NS1 protein expression upon treatment of A549 cells using NIH39 (B) or KH-CB19 (D) with influenza A/WSN/33 infection at MOI 4 for 5 h. Immunoblots are representative of three independent experiments.

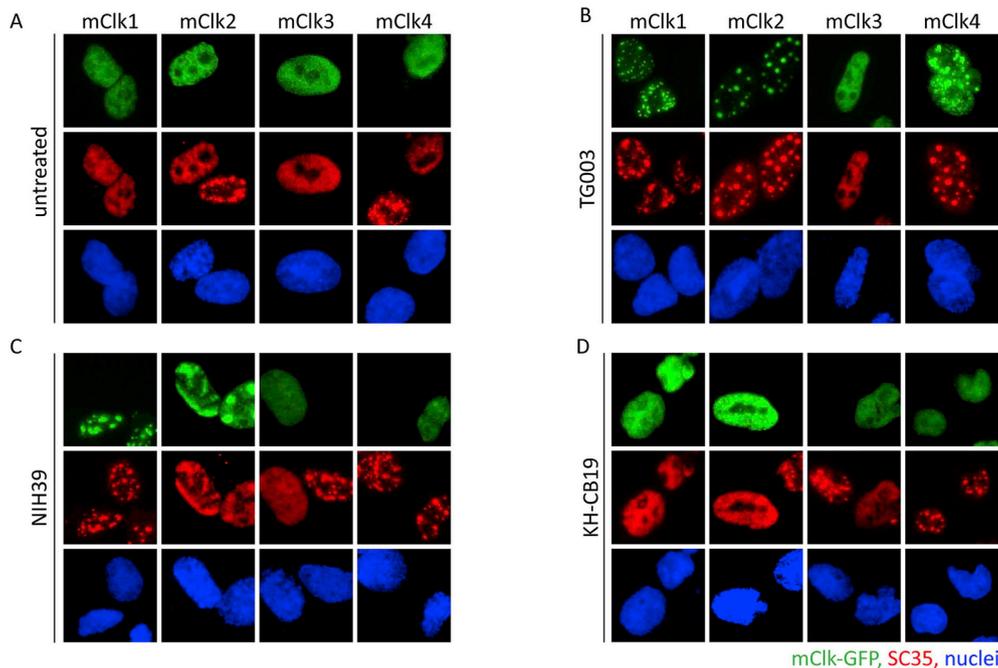


Fig. 5. Treatment with CLK inhibitors alters the consequence of CLK overexpression on SRSF2 (SC35) subcellular distribution. A549 cells were transfected with mCLK-GFP plasmid overnight and then left untreated or treated with 50 μ M TG003, 12.5 μ M NIH39 or 50 μ M KH-CB19 for 6 h. Cells were stained with anti-SC35 antibody and afterwards with Cy3-conjugated goat anti-mouse IgG. Images shown are representative samples of the CLKs and SRSF2 localization pattern from three independent experiments. Fig. S6 provides the quantification of cells showing speckled and non-speckled patterns upon SC35 staining.

2VAG) (Fedorov et al., 2011). For docking simulations, the Schrödinger Suite (<https://www.schrodinger.com>) was used. The compounds were tested against CLK1 in a luciferase-based assay using recombinant CLK1 expressed in *E. coli*, and 56 compounds were found to be effective (Supplementary Table 3). These results were further validated using a capillary electrophoresis LabChip-based enzyme activity/phosphorylation assay capable of determining the IC50 concentration of each substance (Supplementary Table 3). Selected compounds with IC50 values < 1 μ M were then analyzed by virus replication assay in A549 cells to determine antiviral efficacy. We identified two compounds, VCC080174 and VCC463764, which efficiently inhibited

influenza virus replication at very low concentrations (< 1 μ M) and revealed a promising therapeutic index (Fig. 7A and B).

VCC463764 and VCC080174 were then characterized by crystallography to enable further optimization of the chemical structure for maximum effectiveness against CLK1. Solving the crystal structures of CLK1 in complex with VCC080174 to the resolutions of 2.03 Å confirms that the inhibitor is localized within the ATP binding pocket, giving it high efficiency against CLK1 (Fig. 7C). VCC463764 was also found to bind CLK1 at the same binding pocket (data not shown). VCC080174 interacts with CLK1 at the active site by means of several hydrogen bonds (Fig. 7D). The amino group connecting both six-membered rings

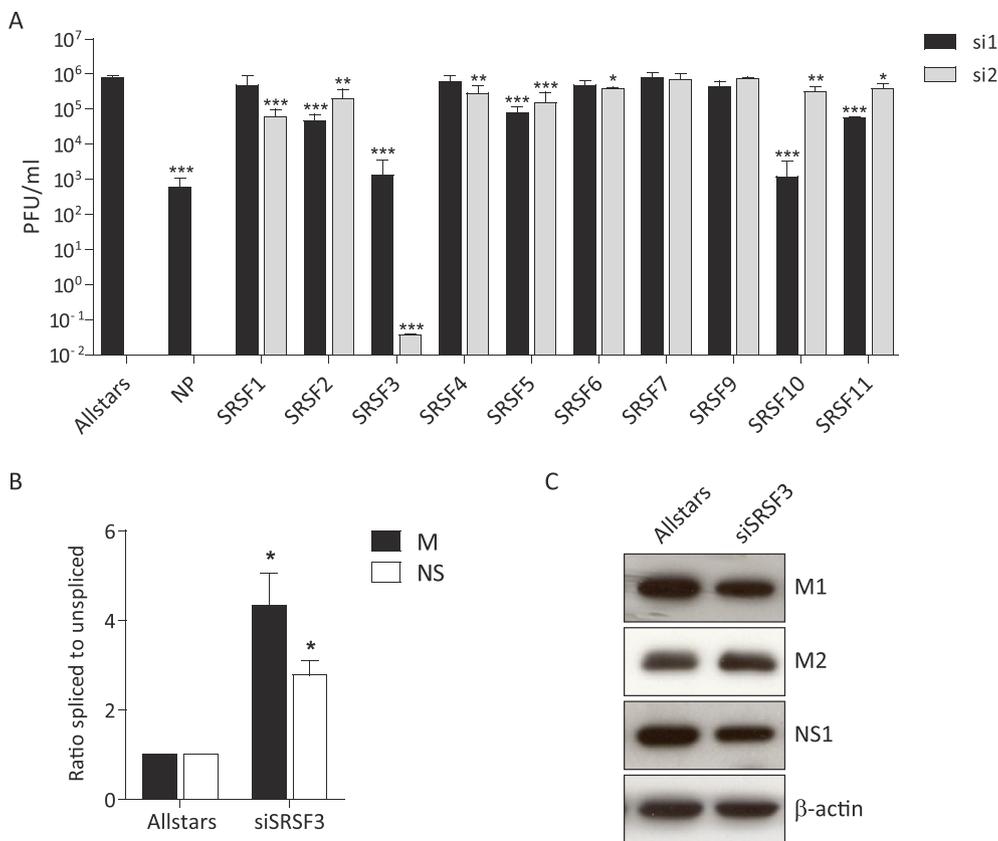


Fig. 6. Identification of downstream targets of CLK1 in correlation with influenza replication inhibition. (A) Two different siRNAs per gene were transfected to A549 cells in 384-well plate format. Replication assay was performed 48 h post transfection by infecting cells with influenza A/WSN/33 (MOI 0.014) for 40 h. Infectious viral particles in the supernatants of infected cells were quantified as PFU/ml. Data represent mean \pm SD of three independent experiments. (B, C) A549 cells were transfected with siRNA targeting SRSF3 or Allstars control in 12-well plate. After 48 h, cells were infected with influenza A/WSN/33 at MOI 4 for 5 h. (B) The ratio of spliced (M2 and NS2, respectively) to unspliced mRNA was calculated by dividing the relative spliced mRNA level by the relative unspliced mRNA level. Results represent mean \pm SEM of three independent experiments; *p-value < 0.05, two-tailed column statistics compared to hypothetical value 1. (C) The influenza M1, M2 and NS1 protein levels upon SRSF3 knockdown were examined using Western Blot. Depicted immunoblots are representative of three independent experiments.

of the inhibitor forms a hydrogen bond with the backbone oxygen atom of Leu 244, whereas the nitrogen acceptor atom in the pyrimidine ring is in close contact to the backbone nitrogen. Further, the thiazole amino group is involved in a hydrogen bond to the Asp 325 carboxyl group. This finding differs from other CLK1 complex structures such as the one with KH-CB19 (PDB code 2vag) showing a different side chain conformation of Asp 325 that enables a hydrogen bond between the carboxylic group of Asp 325 and Gly 327 nitrogen. Similarly, in the isoforms CLK2, CLK3 and CLK4 hydrogen bonds between the corresponding Asp and Gly exist (PDB codes 6fyi, 6fyp, 6fyv). A superposition of the isoform structures suggests that VCC080174 may show a similar hydrogen bond pattern in complexes with other members of the CLK family. In CLK1, Leu 246 is located close to the VCC080174 benzene ring, while in CLK3 this residue corresponds to Lys 241 (PDB code 6fyp). There is no change in CLK2 and CLK4. Further, Val 324 corresponds to Ala 319 in CLK3 whereas there is no substitution in CLK2 and CLK4. Thus, hydrophobic interactions with the thiazole group might be different in CLK3 (Kallen et al., 2018). The similarity of the CLK binding pockets and the identity of key residues, however, suggest that it is difficult to obtain selective inhibitors binding to this site, in particular for CLK1, CLK2 and CLK4.

Substitutions and extensions of the inhibitor structure may enable the targeting of residues that vary between different CLK isoforms. For example, the side chain of Ser 247 is located close to the inhibitor VCC080174 in the CLK1 complex structure. This residue corresponds to Asn 242 in CLK3. Further, Asp 250, corresponding to Glu 245 of CLK3, is located at a suitable distance from the inhibitor. Differential targeting of such isoform-specific key residues may be utilized to optimize inhibitor selectivity during lead optimization.

We then used a cell-based IAV-driven splicing assay that monitors splicing from a viral-like mRNA derived from the M segment to analyze the ratio of viral M1 to M2 mRNA after treatment of HEK293 cells with the two CLK1 inhibitors, using the H3N2 (A/Victoria/3/75) seasonal influenza virus strain. VCC080174 increased the ratio of spliced to

unspliced viral M mRNA (Fig. 7E), similar to the effect observed after inhibition of CLK1 by siRNAs (see Fig. 2A). VCC463764, however, reduced the ratio of spliced to unspliced viral mRNA (Fig. 7D), as was observed with the other chemical inhibitors (see Fig. 4A, C). Despite this, VCC463764 nevertheless impaired virus replication, which suggests that changes in the splicing ratio per se are detrimental to the virus.

4. Discussion

Splicing factors are one of the enriched essential host factors for influenza virus replication identified in several genome-wide screens (Karlas et al., 2010; König et al., 2010; Watanabe et al., 2010). In the screens performed in our lab (Karlas et al., 2016) and by König et al. (2010) CLK1 (Colwill et al., 1996; Prasad et al., 1999), but no other kinases known to regulate splicing, was identified as an essential host factor. Here, we confirm that CLK1 is required for successful IAV replication *in vitro* and *in vivo*, while the other CLK isoforms are not.

We previously found that the CLK inhibitor TG003 inhibits the replication of IAV (Karlas et al., 2010). Based on this initial result, newly developed CLK inhibitors were screened for their effectiveness against IAV replication. Two of the most effective ones, NIH39 and KH-CB19, were further assessed for their effect on influenza mRNA splicing. Only NIH39 enhanced splicing, while KH-CB19 appeared to inhibit splicing. In addition, viral M and NS mRNAs were affected differently. Regulation of alternative splicing is influenced by CLK-mediated phosphorylation of SR proteins. To date, it remains unknown which of the 9 SR proteins are phosphorylated by which of the 4 CLK proteins. Thus, CLK1 might affect the splicing of M and NS mRNA via different phosphorylation status of several SR proteins. We made the same observation when testing two further compounds with enhanced effectiveness against CLK1. Previous reports have shown differential effects of CLK inhibitors towards different CLK isoforms (Muraki et al., 2004; Wong et al., 2011), although the differential role of each isoform remains

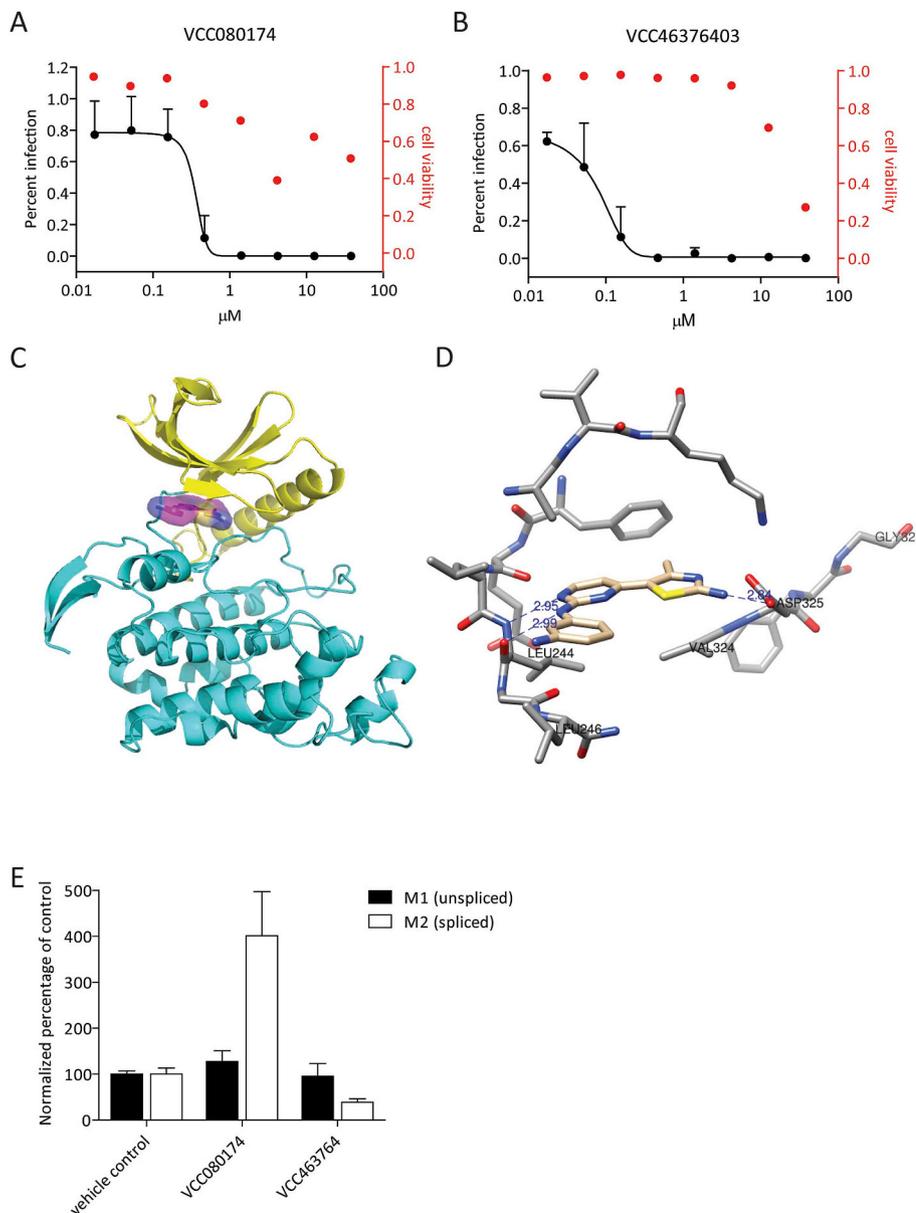


Fig. 7. Dose-response curves performed on A549 cells pre-treated for 2 h with either VCC080174 (A) or VCC463764 (B) and then infected with influenza A virus (A/WSN/33) at MOI 0.01. Cells were further cultivated for 36 h in the presence of these compounds. Red lines indicate cell viability while black lines show reduction in infection level. $EC_{50}(VCC080174) = 0.32 \mu M$, $CC_{50}(VCC080174) = 1.0 \mu M$, selectivity index ($VCC080174$) = 3.1, $EC_{50}(VCC463764) = 0.08 \mu M$, $CC_{50}(VCC463764) = 20.2 \mu M$, selectivity index ($VCC463764$) = 252.5 (C) Crystal structure of CLK1 (the N- and C-lobes are shown in yellow and cyan respectively) in complex with VCC080174 (surface presentation). (D) The close-up shows the hydrogen bonding network between the inhibitor and CLK1 at the binding site (dashed lines). Nitrogen, oxygen and sulphur atoms are plotted in blue, red and yellow, respectively. (E) The influence of VCC080174 or VCC463764 on the splicing of the viral matrix gene segment (M). The splicing rates were analyzed using a dual luciferase assay where Renilla luciferase monitors the unspliced M1 expression levels, and Firefly luciferase indicates M2 expression. The expression of the luciferase reporters was induced by co-infection with the seasonal H3N2 influenza virus strain (A/Victoria/3/75). Results represent mean + SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unclear. Using the subnuclear distribution of SRSF2/SC35 as a read-out, TG003, which effectively inhibits IAV replication, was shown to reverse the effect of CLK1, 2 and 4 overexpression on SC35 subnuclear distribution (Wong et al., 2011, 2013). Here we observed that KH-CB19 was only able to reverse the effect of CLK4 overexpression, while NIH39 showed specificity towards CLK1 and 4. This may explain why NIH39 increases the ratio of spliced to unspliced influenza mRNA in a similar manner to CLK1 knockdown, while KH-CB19 does not. It has been reported before that thermal shift assay experiments with KH-CB19 using different kinases showed the highest temperature shift for CLK4, followed by CLK1 (Fedorov et al., 2011). Meanwhile, the same experiment performed for NIH39 showed the highest temperature shift for CLK1, followed by CLK2 and CLK4 (personal communication). The differences of inhibitor specificity, determined either by using CLK proteins in the thermal shift assay or by using cell based assays as shown in Fig. 5, might be the reason for the different effects on influenza mRNA splicing. These data also suggest that a specific inhibition of CLK1 is required to alter influenza M mRNA splicing. At the same time, CLK inhibitors with a lower affinity to CLK1, but specific for the other CLK isoforms, strongly reduced viral protein expression from both spliced and unspliced mRNA, thus indicating an additional effect on viral

mRNA export or translation, most likely via downstream effects on SR proteins, which also play a role in mRNA export. Several SR proteins, e.g. SRSF1, SRSF7 and SRSF3, can also shuttle between nucleus and cytoplasm and interact with the export factor NXF1 (Caceres et al., 1998; Huang et al., 2004). Remarkably, the biological functions of SR are mainly regulated by distinct phosphorylation states (Botti et al., 2017). Thereby, hyperphosphorylation of SR proteins by CLKs leads to recruitment to transcription sites and spliceosome assembly, whereas the PP1/2A phosphatases-mediated dephosphorylation during splicing causes the release of the splicing machinery and supports the recruitment of NXF1 and thus the export of mature mRNAs (Zhou and Fu, 2013).

Knocking down SR proteins revealed that SRSF3, and to a lesser degree SRSF2 and SRSF5, are essential for influenza infection. The effects of SRSF3 knockdown on IAV mRNA splicing resembled those of CLK1 knockdown. None of these factors have been reported to be involved in influenza infection before, except for co-localization of SRSF2 with the NS1 protein of influenza B virus (Schneider et al., 2009). Interestingly, SRSF1 (SF2/ASF) knockdown only caused a minor reduction of IAV replication, despite the fact that SRSF1 has been shown to bind M mRNA at the 3' exon and regulate its splicing (Shih and Krug,

1996). This does not exclude the possibility that other SR proteins might also regulate M mRNA splicing. SRSF1 has recently also been shown to bind to NS mRNA (Huang et al., 2017), but the interaction of other SR proteins with influenza mRNA sequences and their impact on virus replication still need to be investigated. Also, we cannot rule out that a change in splicing preferences after CLK1 inhibition may lead to an alteration in the global cellular protein composition that could affect influenza virus replication indirectly.

In summary, CLK1 downregulation impairs IAV replication by altering viral mRNA splicing and viral protein expression, most likely via differential effects on SR proteins. For regulation of host cell processes, different CLK isoforms are likely to exhibit some functional redundancy, as no dramatic phenotypical defect was observed in the CLK1 knockout mice. Therefore, CLK1 represents a potential new host-directed target for influenza therapy. By following this rational drug development program, we were already able to greatly improve the efficacy of the CLK1 inhibitors. The next step will be to characterize the ADME profile of these compounds in detail and examine their efficacy against influenza virus infection in animal models.

Data availability

Data are available by request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.06.003>.

Author contributions

AA, YJS, KH, NH, YDE, ND, IS, ML, ZH, OM, RF performed the experiments. OM, RF, WB provided the luciferase splicing assay. KH, NH, FB, LO, GK, IS, ZH provided CLK inhibitors that were further characterized by MN and JvK. YDE, ND, IS, ML, ZH, TD, OL produced recombinant CLK1 and revealed its crystal structure complexed with inhibitors. AA, FB, DE, LO, GK, SG, MN, WB, TFM, AK designed the experiments. AA, MM, MN, JK, TD, DE, OL, OM, RF, WB, TFM, AK analyzed the results. AA, TFM, AK wrote the article.

Conflicts of interest

LO and GK† are owners of Vichem Chemie Ltd; DE, IS and ZH are employees of Vichem Chemie Ltd.

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