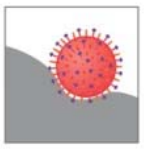


anti flu Innovative anti-influenza drugs excluding viral escape

SEVENTH FRAMEWORK PROGRAMME

Publishable Summary Report

Grant Agreement Number: HEALTH-2010-259842

Project acronym: ANTIFLU

Project title: INNOVATIVE ANTI-INFLUENZA DRUGS EXCLUDING VIRAL ESCAPE

Funding Scheme: Small or medium-scale focused research project (STREP)

Date of latest version of Annex I against which the assessment will be made: 29-06-2016

Period covered: From 01/06/2011 to 30/11/2016

Name, title and organisation of the scientific representative of the project's coordinator:

Prof. Dr. Thomas F. Meyer, Director, **Max Planck Institute for Infection Biology (MPI)**

Tel: +49 30 28460 402

E-mail: meyer@mpiib-berlin.mpg.de

Project website address: http://www.ANTIFLU-project.eu/

Table of Contents

1. Executive summary

Innovative anti-influenza drugs excluding viral escape

Based on a previous genome wide RNAi loss-of-function screen for host determinants involved in influenza virus replication (Karlas, Nature 2010), the ANTIFLU project pursued two major goals: first, it was to prove that influenza infections can be treated therapeutically by targeting host cell factors in vivo. Second, an inhibitor was to be developed, targeting a host cell factor crucial for virus replication but not essential for the host, into a candidate for subsequent clinical development in order to provide the basis for an alternative new influenza therapy. As an important benefit of inhibiting a host cell protein rather than a viral factor, this approach was considered to entirely avoid or at least reduce the risk of pathogen resistance development. To benefit from the outstanding expertise of our partners of the international ANTIFLU consortium a variety of approaches and methodologies was chosen and applied to achieve these goals. On the one hand, small compounds directed against selected kinase targets (VCC, FMP) and non-kinase targets (LDC), were identified by screening different in-house compound libraries and passed through classical drug development including comprehensive validation and optimisation by medicinal chemistry. This process accompanied the genetically engineered production of selected target proteins (HUJI) to allow the set-up of appropriate screening and validation assays. The extensive validation process included antiviral and cytotoxic activity testing (MPI, IC), target-specific functional assays (MPI, IC), co-crystallisation studies to support chemical optimisation (HUJI), and in vivo efficacy studies in mice (MPI) and ferrets (IC). After nomination of a lead compound and further optimisation of that compound, the optimised lead was ready to undergo preclinical development (MT) and end up in a nomination and description of a clinical candidate (IP). In a second approach, selected host cell factors were inhibited by different nucleotide-based strategies (AU, RBT, MPI). This involved the delivery of target-specific siRNAs in combination with nanoparticles as well as the use of naked gapmers (antisense DNA oligonucleotides flanked by locked nucleic acids) which do not require additional in vitro transfection and in vivo delivery tools. Subsequent to chemical modification and in vitro validation of knockdown and antiviral efficacy the most promising gapmers were chosen for in vivo testing.

By the end of the funding period the different approaches had been successfully pursued and resulted in

(1) a promising kinase lead candidate (CLK1 inhibitor),

(2) a nominated non-kinase lead (vATPase inhibitor) which was improved regarding safety margin, metabolic stability and half-life by MedChem and

(3) two gapmers inducing knockdown of vATPase in lung epithelium of mice upon intratracheal application.

In addition, based on the consortiums knowledge on the importance of XPO1 as an influenza antiviral target, two commercially available XPO1 inhibitors were successfully tested regarding their suitability as clinical candidates.

By achieving the crucial milestone of nominating a vATPase lead compound, the general in vivo proof-of-principle of host cell directed therapy was provided. Yet, further improvement of in vivo application will be required to end up in a preclinical candidate. Another important implication for future host-directed therapy was the fact that, in contrast to conventional anti-influenza drugs, neither the CLK-1 lead candidate nor the vATPase lead compound, induced resistance development of influenza virus in vitro. Finally, the gapmer-mediated knockdown of the vATPase gene expression in mice provided an excellent basis for further improvement of the knockdown rate and the inhibition of viral replication in vivo.

Aspects of all the results obtained have been comprehensively summarised for a number of intended publications as well as an international patent application.

In addition to the listed partners, the continuous active support of IP as an advisory body related to influenza research and public health issues is to be noted here and, similarly, the consultancy of MT with regard to *in vivo* toxicity studies. Finally, ART supported the coordinator in all aspects of project management, in particular the financial management of the ANTIFLU project.

2. Project context and main objectives

Project context

The aim of the ANTIFLU project was to develop new and alternative drugs against influenza by exploiting the crucial function of host cell determinants as targets of small molecules and RNAi inhibitors in order to prevent viral infection and growth. This promising new approach should open the route to new and alternative treatment options to combat influenza, which have the potential to complement currently available strategies and overcome their limitations, such as resistance development and viral variability.

Viral replication is known to depend on multiple host cell factors. Whilst traditional anti-influenza treatments usually target viral factors, ANTIFLU pursued a host-oriented approach, focusing on translational research to identify drugs that interfere with host response pathways and host cell determinants that play a role in the development of the disease. ANTIFLU was built upon an existing repertoire of antiviral targets resulting from an RNAi-based genome-wide influenza virus screen carried out by the coordinator of the ANTIFLU project (Karlas A, Machuy N. et al., Nature 2010, 463:818-22). The interdisciplinary consortium intended to identify and select validated host cell targets and druggable lead compounds (kinase and non-kinase ligands) active against them, refine them by medicinal chemistry into clinically applicable drugs, and perform preclinical studies. In addition, crucial host cell functions not targeted by conventional drugs were going to be explored using oligonucleotide-based approaches. Therefore, knowledge generated in the former EU FP6 project RIGHT was employed to produce potent target-specific nucleotides and apply advanced methods for their delivery. This approach is likely to present an answer to recently emerged needs in anti-influenza strategies, since it should avoid the well-known pitfalls of resistance formation while remaining suitable for broad application. In case of the seasonal strain variations, such a drug could be readily used without the necessity for detailed diagnosis before drug treatment begins. In addition, antivirals targeting host determinants could be used for avian and swine influenzas, for a potential influenza pandemic as well as for stockpiling.

Main Objectives

The aim of the ANTIFLU project is to develop novel drugs against influenza that target key host-cell determinants with small molecules and RNAi inhibitors in order to prevent viral infection and replication. This promising new approach has the potential to complement currently available strategies and overcome their limitations, such as those posed by resistance and viral variability.

The main goals of the ANTIFLU project were:

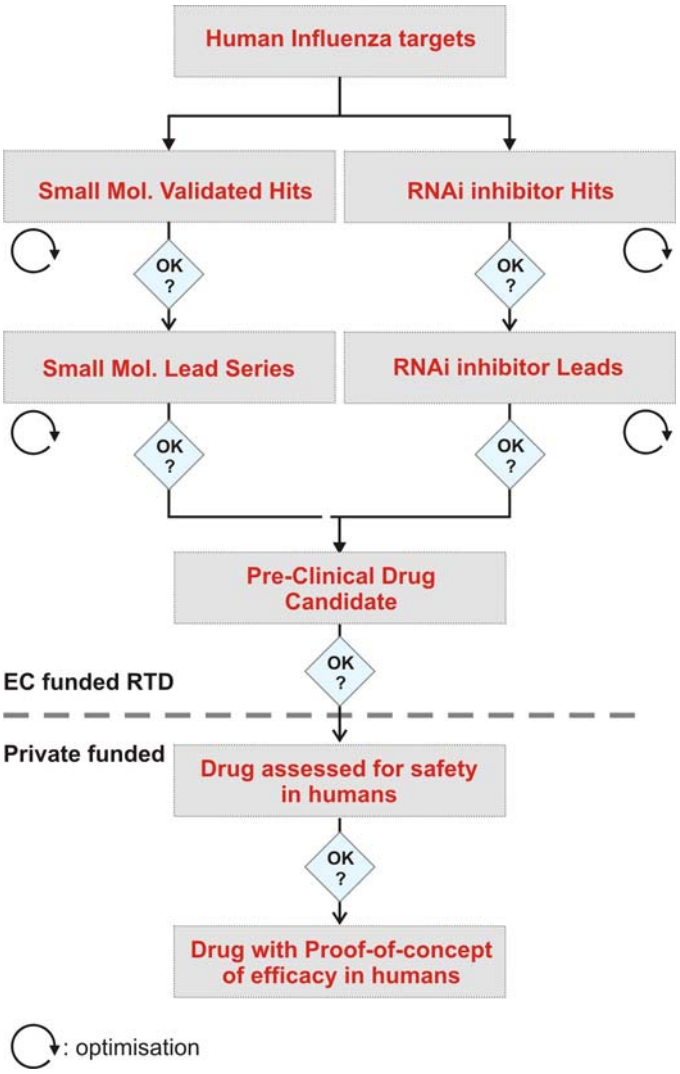
- The proof of principle that influenza infection can be efficiently treated by targeting human determinants on either protein or RNA level, using small molecule or siRNA inhibitors,

  and

- A novel therapeutic strategy to combat influenza virus infections by more failproof (avoidance of resistance) and more versatile means (broad intra-species spectrum)

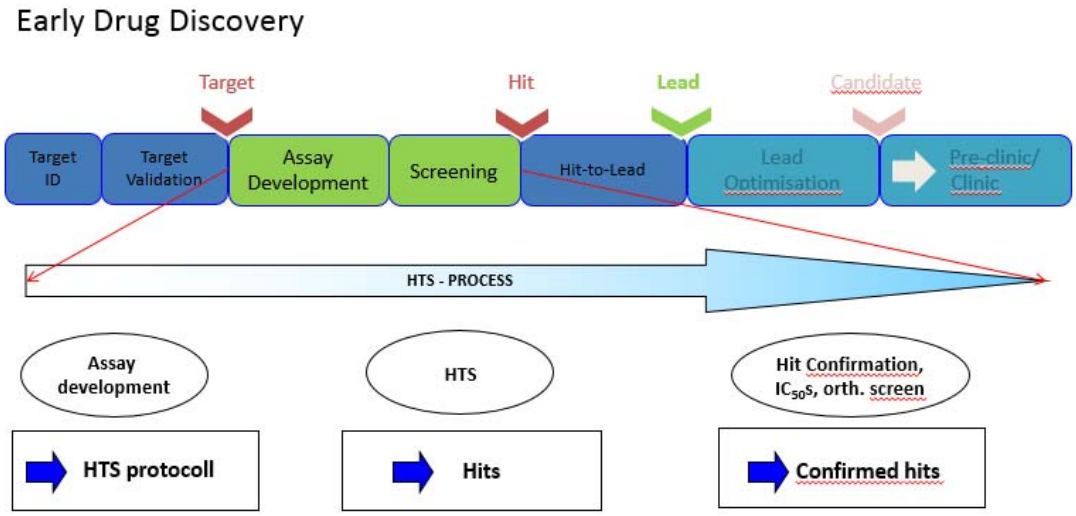
Research Plan

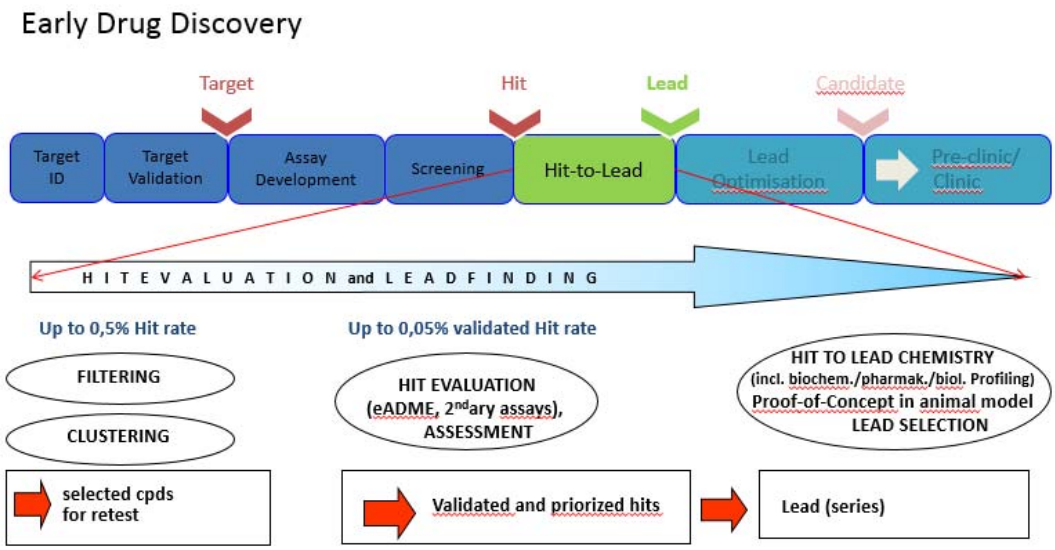
The ANTIFLU project built upon two main therapeutic approaches: small molecule inhibitors and RNAi-based inhibitors, both targeting human factors. The interdisciplinary consortium identified and selected validated host-cell targets, selected druggable lead compounds (kinase and non-kinase ligands), aimed to refine these compounds into clinically applicable drugs, and to perform preclinical developments. In addition, host-cell functions crucial for viral replication, which are not targeted by conventional drugs, were supposed to be explored using therapeutic RNAi. Therefore, knowledge generated from the past FP6 project, RIGHT (http://www.ip-right.org/), was used to produce potent RNAi inhibitors and apply advanced methods for the delivery of siRNA. On completion of the ANTIFLU project clinical studies through private funding were supposed to follow. A scheme of the drug development and a description of the two main branches is given below:



Small molecule inhibitors

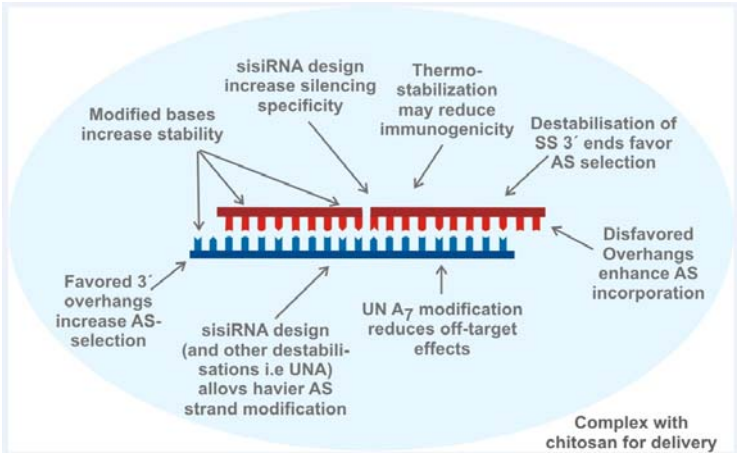
A major problem facing current influenza drug therapy is increased resistance to current small molecule compounds, e.g. M2 inhibitors (Amantadine and Rimantadine) and neuraminidase inhibitors (Zanamivir and Oseltamivir). Thus, it is evident that novel, broad-spectrum small molecule-based drugs directed against human targets involved in influenza infection are urgently needed. The modulation of host cell proteins that are essential for virus replication might antagonize virus propagation and could provide both prophylactic and therapeutic options. One major focus of the ANTIFLU project was therefore the discovery and further development of specific small molecule ligands acting on host cell targets. This strategy placed high demands on safety to avoid target-mediated toxicities, which were met by using the highest industrial standards and hard milestone criteria. The individual steps of early drug development are illustrated below:





RNAi based inhibitors

RNAi-mediated host gene targeting can be used to inhibit any host cell factor, including those that have so far been non-druggable, and thereby significantly expands the options for possible therapeutic compounds and strategies. The major advantage of RNAi-based antiviral therapy in comparison to classical small compounds, such as conventional kinase inhibitors, is the very high specificity of siRNAs. Apart from (i) achieving maximum on-target activity, (ii) avoiding off-target effects, (iii) suppressing immunostimulatory potential, and (iv) circumventing competition of siRNAs with endogenous miRNAs for essential cellular factors, the major challenge facing an RNAi-based approach was to overcome extracellular and intracellular barriers to nucleic acid delivery; for example, by using cationic polymers (polyplexes) or encapsulated polymeric nanoparticles and others (see below).



Consortium and Funding

The ANTIFLU project (GA No 259842) was funded by the EC FP7 Programme with 6 Mio € for 5.5 years and included 5 SMEs, internationally renowned research groups and clinical institutions experienced in anti-influenza treatment and clinical trials.

Max Planck Institute for Infection Biology (MPI), Berlin, Germany, Prof. Thomas F. Meyer, Dr. Alexander Karlas

Vichem Chemie Research Ltd (VCC), Budapest, Hungary, Prof. György Kéri, Prof. Lázló Orfi, Dr. Daniel Eros

Aarhus University (AU), Aarhus, Denmark, Prof. Jørgen Kjems, Prof. Ken Howard

Lead Discovery Center (LDC), Dortmund, Germany Dr. Bert Klebl, Dr. Klaus Dinkel

Institut Pasteur (IP), Paris, France, Prof. Marc Lecuit, Dr. Cécile Delval, Dr. Cécile Artaud

ARTTIC (ART), Paris, France, Rémi Beteille, Andreas Schweinberger

MediTox s.r.o (MT)., Konárovice , Czech Republic Dr. Martin Slais

Imperial College London (IC), UK, Prof. Wendy S. Barclay, Dr. Olivier Moncorge

RiboTask (RBT), Odense, Denmark Prof. Jesper Wengel

The Hebrew University of Jerusalem (HUJI), Israel Prof. Oded Livnah, Dr. Tsafi Danieli

Leibnitz-Institut für Molekulare Pharmakologie (FMP), FMP Berlin Dr. Jens Peter von Kries

Coordination: Prof. Dr. Thomas F. Meyer supported by Dr. Sigrid Gödert, Max Planck Institute for Infection Biology, Department of Molecular Biology, Berlin, Germany, (antiflu-mey@mpiib-berlin.mpg.de)

3. Description of the main results/foregrounds

Target Selection (WP1)

The approach taken by the consortium builds upon a panel of human targets previously identified in the course of a genome-wide RNAi screen for human factors essential for influenza virus replication *in vitro (Karlas A, Machuy N et al., 2010)*. This screen of about 62,000 siRNAs targeting around 17,000 annotated genes and 6,000 predicted genes revealed 287 human factors involved in the replication of influenza virus and subsequent validation finally resulted in 168 hits (Fig. 1).

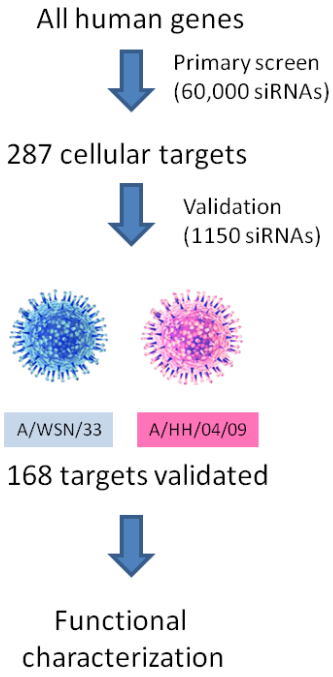


Fig. 1 Schematic outline of the primary screen and the validation procedure.

The composition of targets was analysed using the Ingenuity software (www.ingenuity.com/). Interestingly, many targets are classified as kinases, phosphatases, ion channels, proteases or enzymes in general and these factors belong to the class of so-called druggable targets, because they can be addressed by small molecule inhibitors (Fig. 2)

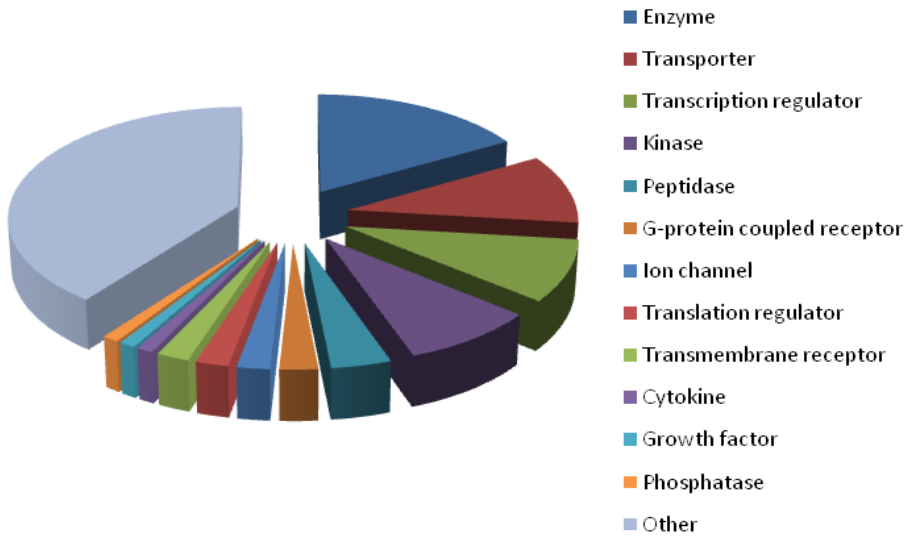


Fig. 2 Target composition of the cellular host cell factors. The identified host cell genes identified in the genome wide RNAi screen were further classified according to their types using the software package Ingenuity (www.ingenuity.com).

To narrow down the number of promising target candidates, all identified hits derived from the RNAi screen were filtered by looking at the following aspects:

- Strengths of the antiviral effects based on MPI's own screening data
- Results in related screens (Brass et al., 2009; Hao et al., 2008; Konig et al., 2010; Shapira et al., 2009)
- Location and type of the target protein
- Strength of expression (in infected and non-infected cells)
- Toxicity (own data and data from the DKFZ, Heidelberg, (Gilsdorf et al., 2010)
- Availability in the PDB (according to the protein data base)
- Transgenic mice and corresponding phenotype
- Availability of chemical inhibitors
- Patent situation

All relevant data were collected and 84 targets that fulfil the criteria listed below were pre-selected:

(i) essential for influenza virus replication
(ii) dispensable (at least short-term) for the host
(iii) classified as druggable protein
(iv) not interfering with innate immunity pathways
(v) no patent conflicts are foreseeable

In an additional validation round three different parameters were addressed (Fig 3):

(i) effects on virus replication upon gene knockdown
(ii) determination of knockdown efficacy
(iii) toxic effects due to knockdown
(iv) interferon induction upon knockdown

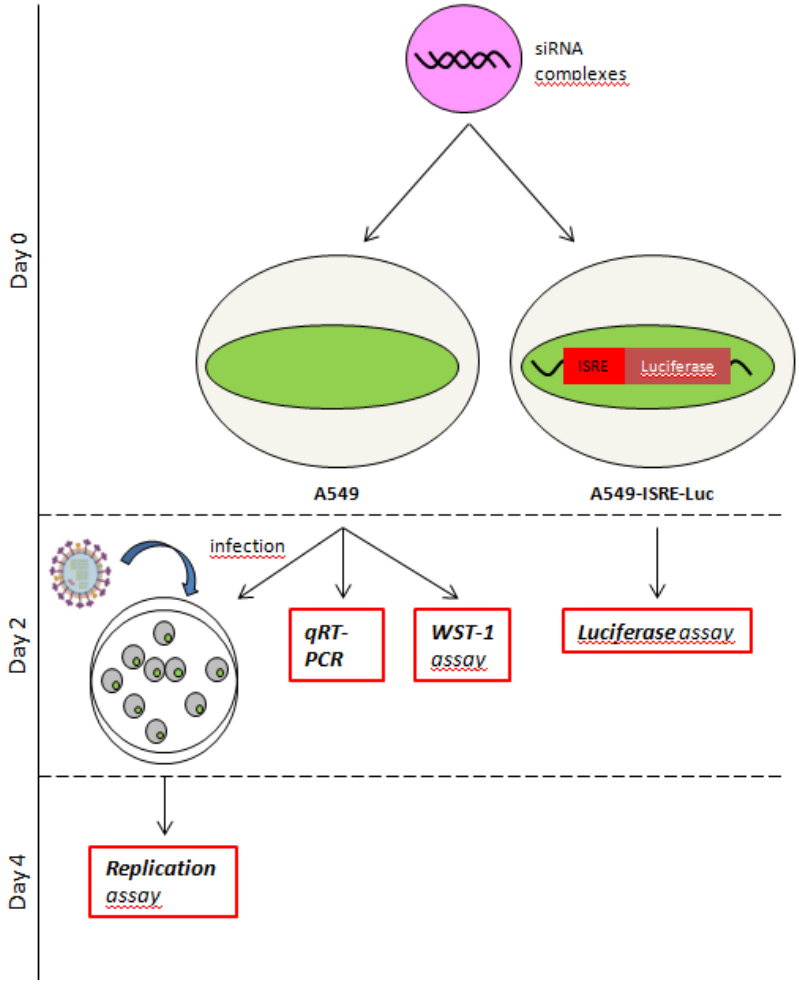


Fig. 3 **The outline of the second target validation by siRNA screening.** During this approach the effect of gene knockdown on virus replication (Replication assay), cytotoxicity (WST assay), interferon response (Luciferase assay) and target knockdown (qRT-PCR) of all individual siRNA sequences was analysed.

In addition, where possible, commercially available drugs were tested for antiviral activity in vitro. All relevant data on the target hits gained by comprehensive bioinformatics analyses followed by experimental validation were integrated into a "validation score" by the development and implementation of a sophisticated method for filtering and ranking the target hits. The most suitable kinase and non-kinase targets as well as targets for inhibition by siRNAs were selected from this ranked list of target genes by MPI/LDC in close collaboration with the ANTIFLU partners VCR, IC, FMP, HUJI and AU (MS1).

In summary, CLK1 was nominated as main kinase target and vATPase as main non-kinase target (for description of the targets see below). Whereas CLK1 is a splicing factor that modulates splicing in the host cell, which probably also leads to a misbalance of spliced and un-spliced viral mRNAs, vATPase is responsible for the acidification of endosomes, a prerequisite for the release of virus RNA into the cytoplasm. For the RNAi approach 7 target proteins were selected.

Small compound approach (WP2, WP3, WP4,WP7))

Assay development, high throughput screening and hit validation (WP2)

High throughput screening of substance libraries for the selected targets necessitated the production of several targets, as well as the setup of target specific assays. Therefore, in a first step the prioritised kinase and non-kinase target proteins were either recombinantly produced and purified by HUJI, or obtained from other research groups or companies. Recombinant CLK1 was cloned, produced and purified by HUJI for identification of CLK1-specific compounds at FMP and VCR. Quality-controlled vATPase multi-protein complex was provided by Wieczorek/Huss (University of Osnabrück/Germany) for identification of vATPAse-specific compounds at LDC. For Subordinate screening campaigns against XPO1 and PLD2 at LDC recombinant XPO1 was generated by HUJI and recombinant PLD2 was obtained from commercial suppliers. For kinase NEK9 screening, recombinant NEK9 protein was provided by Stefan Knapp (University of Oxford/UK), but additional amounts had to be obtained from commercial suppliers.

In a second step, appropriate assays for high throughput screening of the individual targets were developed and screens carried out, using the libraries of the partners FMP, LDC and VCR, which comprised 35,000, 200,000 and 1,363 compounds, respectively. Utilizing these target proteins, the following HTS biochemical assays were developed: based on an ADP-Glo® kinase assay VCR (Fig. 4), successfully set up a HTS assay against CLK1 as well as a TranScreener assay for mTOR; FMP established a comparable screening assay against CLK1 and NEK9 by using capillary electrophoresis on a microfluidic chip system (LabChip-3000, Fig.5); LDC used purified vATPase protein complex to set up a screening assay against the vATPase holoenzyme V1/VO by adapting the ADP-Glo® kinase assay (Fig. 4) and established a counter screen assay for vATPase subunit enzyme V1. For the secondary screening project (PLD2) at LDC a MTS biochemical assay was developed. HTS resulted in 26 highly active CLK1 compounds at VCR, 120 CLK1 hits at FMP with activities in the 20 nM to 1 µM range and 230 primary hits against vATPase identified at LDC. For the backup targets MAP2K3, ACK-1, NEK9, mTOR and XPO1, suitable assays were developed by VCR, FMP and LDC and screens performed. XPO1 has been screened in silico by LDC.

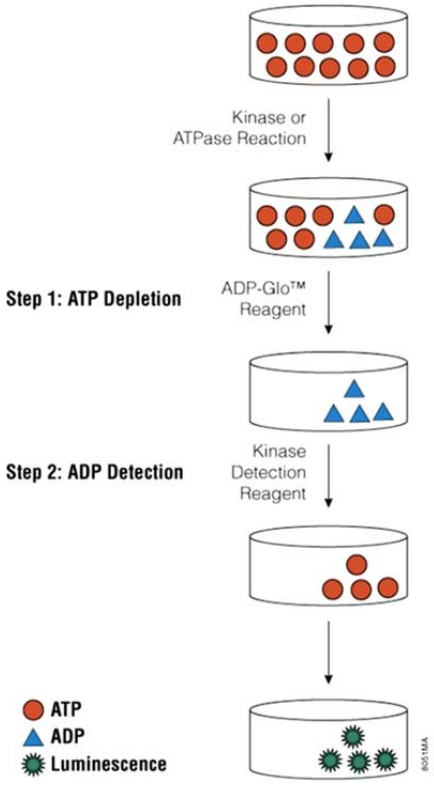
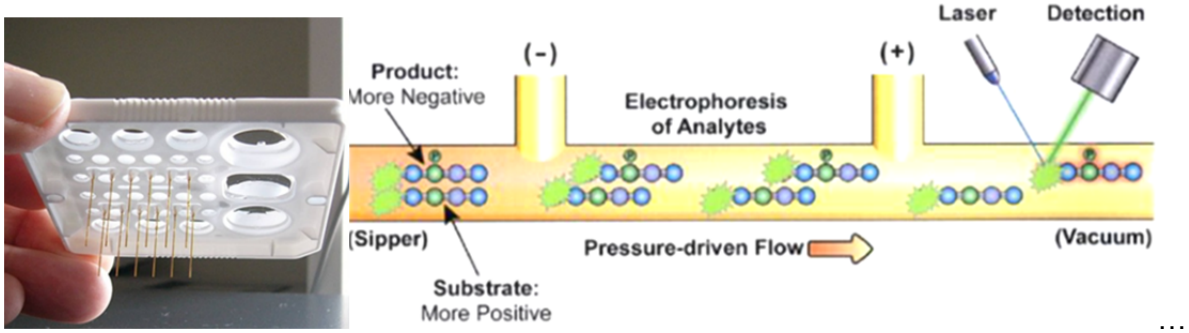


Fig 4 ***ADP-Glo® kinase assay.*** *The ADP-Glo™ Kinase Assay is a luminescent ADP detection assay that provides a universal, homogeneous, high-throughput screening method to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction.*



…

Fig 5 ***Capillary electrophoresis in a microfluidic chip system (LabChip-3000).*** *By applying a vacuum nanoliter amounts of enzyme reaction solution are transported within 12 sippers at bottom of the chip and transported through 12 separate microfluidic channels in electrophoresis buffer. Phosphorylated and non-phosphorylated peptides are separated in the electrophoretic field (+/- poles). Fluorescent labels covalently linked to the peptides are excited by a laser and their emission detected by a highly sensitive ccd-camera.*

The resulting hits were subsequently tested for cellular efficacy in an in vitro influenza replication assay at MPI, followed by further validation steps using newly established orthogonal assays and cell viability assays to determine side effects and safety, respectively. An orthogonal cellular vATPase assay was developed and validated by LDC (cell-based Lysotracker assay) and for CLK1 an IMAP assay was established by VCR. Thirteen vATPase hits with confirmed enzymatic IC50 values of < 1 µM were validated in the influenza replication assay (MPI), in vitro toxicity assays (WST-1 by MPI, Cell Titer-Glo® by LDC) and an orthogonal Lysotracker imaging assay (LDC). Following selection and validation in cellular assays (functional and phenotypical) hits were nominated.

Partners mainly involved (and their main task):

VCR (assay development, compound screen, hit identification and validation)

HUJI (target production)

MPI (in vitro validation by influenza replication assay and WST assay)

LDC (assay development, compound screen, hit identification and validation)

FMP (assay development, compound screen, hit identification and validation)

Hit optimisation - hit to lead (WP3) and lead optimisation (WP4)

Following the hit nomination, hit validation was completed and the validated hits underwent chemical optimisation to yield pharmaceutically active substances, so-called leads, with potent activity on the target (nanomolar biochemical affinities), and antiviral activity in vitro (influenza replication assay) and in vivo (influenza mouse models). The chemical optimisation of validated hits was achieved by different approaches, e.g. synthesis of new derivates, SAR (structure activity relationship) generation, computer-assisted drug design and co-crystallisation with the target protein. In iterative cycles the nominated lead series were optimised not only for potency against the target, but also for physiochemical and pharmacological properties to achieve good application to and tissue distribution in animals. In the following part the main results of the lead nomination and lead optimization are described for the kinase hit CLK1 and the non-kinase hit vATPase separately.

Kinase inhibitor CLK1:

A focused set of compounds for screening against CLK1 was assembled at VCR. Reference compounds were synthesized and profiled to validate assays and SAR were studied. Tools for computer-aided drug design of CLK1 inhibitors were tested and validated. A CLK1 pharmacophore model was developed to allow virtual docking simulations. Based on experimental and *in silico* data, CLK1-specific compounds were selected for co-crystallization experiments. Numerous expression conditions and purification protocols were developed, which finally resulted in crystals used for structure determination of CLK1 complexed with small molecule lead inhibitory compounds. The validation of selected hits for antiviral activity by influenza replication assay at MPI was performed in standard cell lines, and in part in primary human airway epithelial cells to measure influenza replication after compound treatment under more physiological conditions. In addition, a CLK1 knockout mouse model was further improved to achieve a better *in vivo* knockout and confirm the antiviral potential of CLK1 knockdown *in vitro* and *in vivo*. At IC CLK1 inhibitors were tested in MDCK and Calu-3 cell lines for influenza replication and a reporter assays was developed to analyze the splicing events modulated by CLK1 inhibitors. Furthermore, an in situ polymerase assay was established to test the influence of chemical inhibitors on viral polymerase. A resistance assay to assess the potential risk of inducing escape mutations upon treatment with the kinase inhibitors was developed at IC by genetic modification of influenza virus through a single mutation within the M2 gene segment, making this virus sensitive to adamantanes (Amantadine and Rimantadine) again. By passaging the virus-containing supernatants to freshly seeded cells, the natural spread of virus infection was simulated. CLK1 inhibitors did not lead to resistant IAVs

after six passages in cell culture. Apart from that, the co-crystallization studies with complexes of CLK1 and different CLK1 inhibitors resulted in a total of 57 identified complexes with CLK1 inhibitors and 83 three-dimensional structures of CLK1. Taken together, based on 26 hits with enzymatic IC50 values of < 5 µM identified by VCR, three synthesis cycles (58 cpds– 32cpds – 24cpds) were performed and CLK1 hits were characterized in depth with the enzymatic assays ADP-GLO® (VCR) and LapChip3000 (FMP), enzymatic IMAP assay (VCR), influenza replication assays (Fig.6, MPI, IC), *in vitro* toxicity assays (VCR, MPI), reporter assays for splicing (Fig.7, IC) and co-crystallization analyses (Fig.8, HUJI). In parallel the CLK1 target was further validated in Clk1 knockout mice (Fig.9), cells derived from these mice (Fig.10), CRISPR/Cas9-based CLK1 knockout cells (Fig.11) (MPI) and primary cell models (IC, MPI). Some of the results are presented in the following figures:

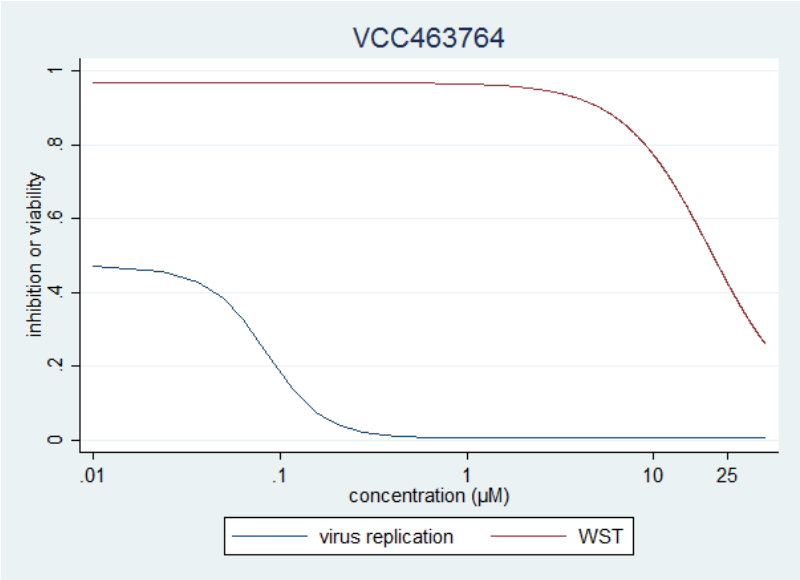


Fig. 6 ***Dose response curves of the CLK1 inhibitor VCC463764.*** *A549 cells pre-treated for 2 h with VCC463764 (B) and then infected with influenza A virus (A/WSN/33) were further cultivated for 36 h in the presence of this compounds. Red lines indicate cell viability while black lines show reduction in infection level.*

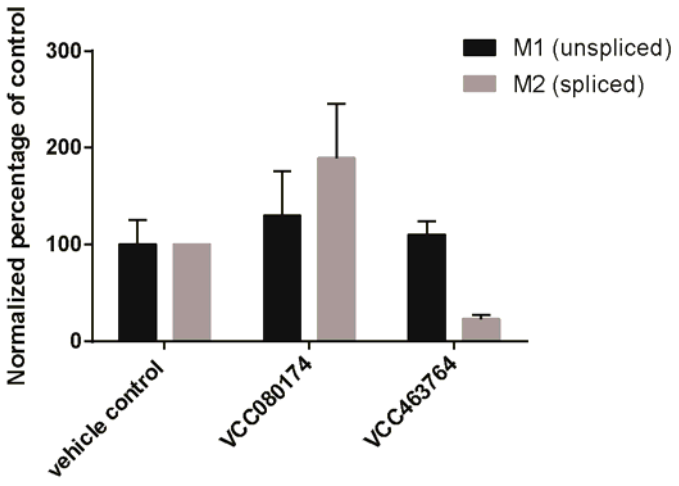


Fig. 7 ***The influence of VCC080174 or VCC463764 on the splicing of the viral matrix gene segment (M).*** *The splicing rates were analysed using a dual luciferase assay where Renilla luciferase monitors the unspliced M1 expression levels, and Firefly luciferase indicates M2 expression.*

Fig. 8 ***Crystal generated for structure determination of CLK1.*** *Purified CLK1 was complexed with small molecule lead inhibitory compound.*

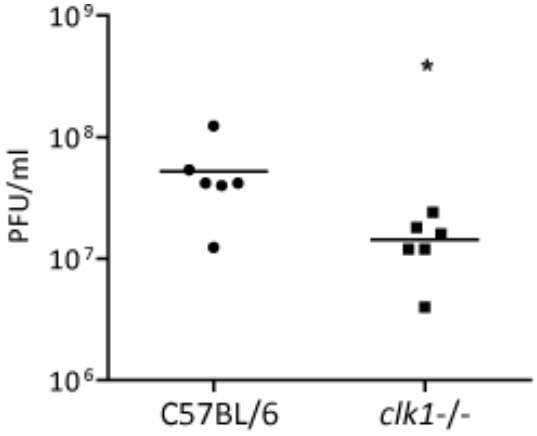


Fig. 9 ***Viral load in the lungs of wildtype and CLK1-/- mice.*** *C57BL/6 wild-type or CLK1-/- mice (n=6) were intranasally infected with 9x10^4 PFU of influenza A/England/195/2009 (H1N1). At 48 h after infection, infectious viral particles within the lungs were quantified using plaque assay. *p-value<0.05, two-tailed unpaired t-test.*

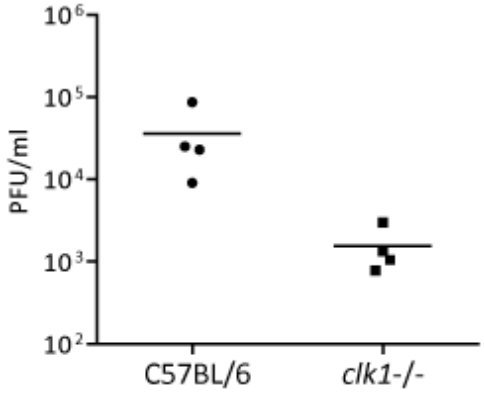


Fig. 10 ***Viral replication in cells from wild-type or Clk1-/- mice.*** *Cells isolated from the lungs of either C57BL/6 wild-type or Clk1-/- mice were infected with influenza A/WSN/33 at MOI 0.01 for 72 h. Production of infectious viral progeny was measured using indirect immunofluorescence staining.*

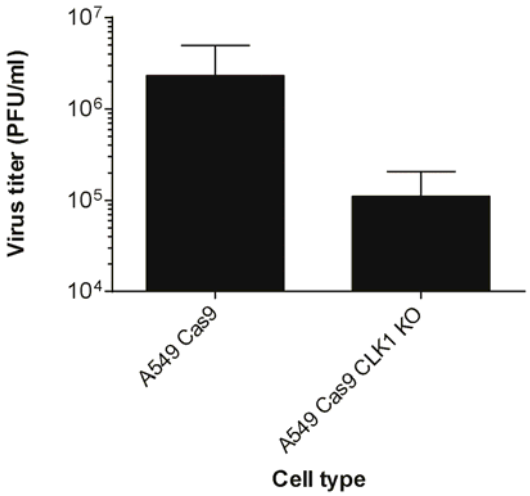


Fig.11 Viral replication in cells depleted for CLK1 by CRISPR/Cas9. *Amount of virus produced in A549 cells depleted for CLK1 by CRISPR/Cas9 and infected with A/WSN/33 at MOI 0.001 for 24 h. Virus titer was quantified by plaque assay (shown are mean values of three independent experiments).*

Most promising was a hit series of Hesperadin analogues which had been further developed and resulted in a lead candidate (VCC463764) that showed enzymatic and antiviral activity in the nanomolar range *in vitro,* whereas cellular toxicity was observed only at micromolar concentrations. After kinase selectivity testing and ADME characterization two in vivo PK studies were assigned and completed. A third PK study to define a well-tolerable dosage for an *in vivo* efficacy study with influenza-infected mice could not be performed within the given project duration. Thus the final proof-of-concept study to clarify whether VCC463764 fulfills the criteria of a lead compound is pending.

In summary, a promising CLK1 lead candidate was selected for which an in vivo efficacy study could not be performed in the frame of the project. Two manuscripts about CLK1 were prepared for submission: one about the method established for recombinant expression of CLK1 and the corresponding co-crystallisation studies and another about the functional characterisation of CLK1. Noteworthy, the structure and functional features of the most active Hesperadin analogues were protected in the frame of a European patent application, which was filed in February 2015 (EP3056202 – Benzopyrolidone derivatives possessing antiviral and anticancer properties) followed by an international patent application (PCT) in February 2016 (WO2016/131789 A1).

Partners mainly involved (and their main task):

VCR (hit validation and optimisation)

HUJI (CLK1 production and co-crystallization studies)

MPI (in vitro validation by influenza replication assay, WST assay, KO mice, CRISPR)

IC (compound validation by splicing and polymerase assay)

LDC (determination of ADME parameters and design of PK studies)

FMP (validation by LabChip-3000)

Non-kinase inhibitor vATPase:

Based on the vATPase hit series the SAR (structure activity relationship) was generated and a correlation between the target vATPase assay and the viral replication assay was proven, confirming the working hypothesis of vATPase as host anti-influenza target. ADME parameters (plasma protein binding and microsomal stability) were determined and improved. LDC generated validated hits and gained sound knowledge on SAR and SPR. The single steps performed from screening to lead generation are illustrated in Figure 12.

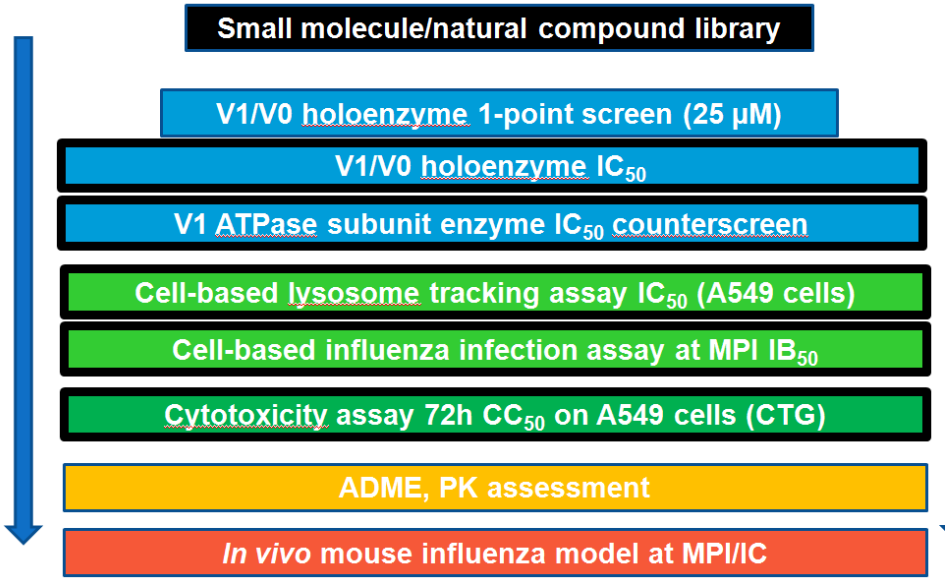


Fig. 12 Assay cascade
Screening – Hit nomination – Hit refinement – Lead generation

This knowledge was used to generate a series of sub-micromolar hits with acceptable ADMET properties. The hits were active in cells in mechanistic as well as functional assays. Frontrunner compounds were selected according to the lead criteria defined in the work plan and forwarded to pharmacokinetic and tolerability studies in mice. Pharmakokinetic profiles, single and repeat dose escalation studies, as well as assessment of lung exposure were performed. Several compounds showed systemic accumulation, as well as a particular concentration increase (up to 10 µM) in the lung. vATPase lead candidates were validated by an enzymatic assay (LDC), an orthogonal lysotracker assay (LDC), two in vitro toxicity assays (LDC, MPI), a virus replication assay (MPI), pharmacokinetic and tolerance studies in mice (external) as well as proof-of-concept studies in a murine influenza infection model (MPI). Apart from that, resistance assays established and performed for the vATPase lead inhibitor by IC did not lead to resistant IAVs, supporting the idea that host-directed therapy approaches reduce the risk of resistance development. In month 43, as a major goal of the project, a lead (LDC193273) could be nominated according to the pre-defined lead criteria (Fig.13, 14) and thereby an important and crucial Milestone was achieved.

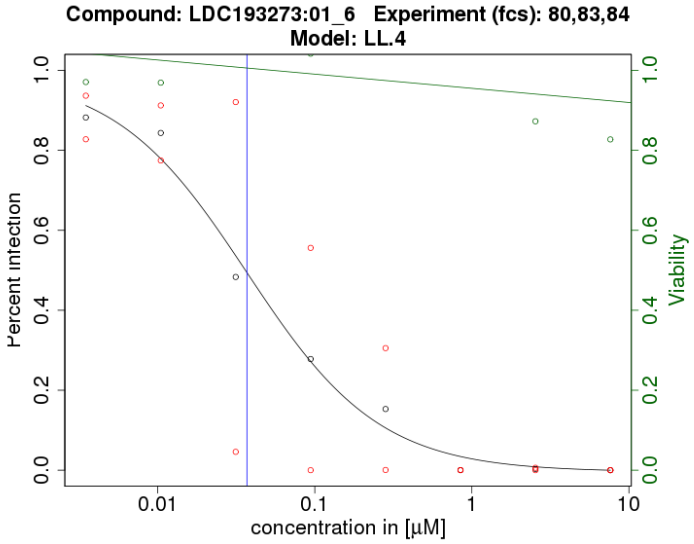


Fig. 13 The dose response curve of the vATPase inhibitor LDC193273 tested in vitro. *(Reduction of viral replication is shown in black, viability of the cells in green)The curve fitting algorithm predicts an IC50 concentration of 40 nM.*

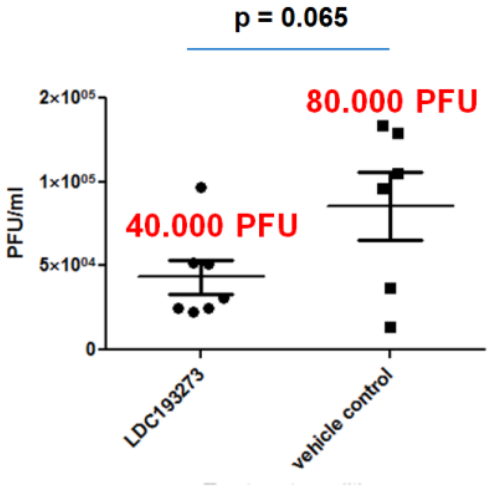


Fig. 14 In vivo POC testing of LDC193273 after repeated dosing. *(100 mg/kg po, animals were treated once a day.)*

Subsequently, MedChem was used for lead optimization, accompanied by continuous *in vitro* and *in vivo* validation. The optimisation of the vATPase inhibitor lead compound was completed in the frame of two iterative cycles including the synthesis of derivatives, *in vitro* profiling and *in vivo* PK studies. Within the lead optimization, LDC was able to deliver compounds that were significantly improved in terms of

- Safety margin *in vitro* (Toxicity EC50 / Efficacy IC50)
- Metabolic stability in mouse and human liver microsomes (ADME)
- *In vivo* PK (t ½ time)

Despite the improvement of the PK/PD modelling profiles, the optimized leads (LDC192593 and LDC201460) unexpectedly failed to show statistically significant efficacy (reduction in viral lung titers) in the mouse influenza model after oral administration. Further improvements of critical

parameters (AUC; Clearance; high Vd) could not easily be addressed by MedChem modifications. Therefore two additional application routes, intranasal and oropharyngeal, were tried. Although intranasal application resulted in a significant reduction of viral load in the lungs of infected mice, the effect was too weak in comparison to the reference drug to justify a further development of the compounds. Oropharyngeal administration resulted only in a minor effect on viral load in the lungs.

In summary, the vATPase inhibitor compound LDC193273 fulfilled all the pre-defined lead criteria (sub-micromolar in vitro and in vivo activity) and was thus nominated as lead molecule for the non-kinase inhibitors. Lead optimization by MedChem yet failed to result in a clinical candidate.

Partners mainly involved (and their main task):

LDC (refinement, synthesis and *in vitro/in vivo* profiling of new derivatives)

MPI (compound validation by *in vitro* influenza replication assay and WST assay)

IC (resistance assays)

Non-kinase backup target XPO1 (WP4, WP7)

Exportin 1 (XPO1) was identified by RNAi screening as an important host cell target in the influenza model and selected as a backup target based on chemical validation by the Streptomyces metabolite XPO1 inhibitor leptomycin B (LMB) as reference XPO1 inhibitor. LDC established a binding assay to perform a virtual screen, leading to several hits. Due to the poor profile (low potency, ADME liabilities) of the identified hits, however, chemical optimization was not recommended. Instead, a commercially available compound turned out to be a top candidate XPO1 inhibitor with very low IC50 values in the binding assay and antiviral activity assay. Therefore, a possible joint development of this drug for influenza therapy in the frame of the ANTIFLU project was evaluated and negotiations with the owner company were initiated (not all data can be disclosed here). After a CDA was signed, a test for a biomarker was established and confirmed *in vitro* and *in vivo*. In addition, the *in vivo* efficacy was confirmed, as oral treatment with the compound resulted in a strong reduction of the viral load in the lungs of influenza-infected mice. In summary, the pre-clinical testing of the compound was completed, but finally no agreement with the owner company could be achieved.

Partners mainly involved (and their main task):

LDC (assay development, *in vitro* validation, negotiation with the industrial partner)

MPI (target engagement testing *in vitro* and *in vivo, in vivo* efficacy study in mice)

IC (limited PK study in ferrets, efficacy study in ferrets)

RNAi approach (WP6, WP7)

The major aims of this branch of the ANTIFLU project were firstly to validate siRNAs against influenza virus-relevant targets, and secondly to establish an *in vivo* siRNA delivery system that can be used to reduce the viral load in the lungs of influenza-infected mice and thereby development an RNAi-mediated therapeutic approach for influenza infection.

siRNA hit design, synthesis and in vitro validation (WP5)

Since different cell and animal models existed within the consortium, siRNA sequences for the three species (human, mouse, pig and initially also ferret) were designed (AU, RBT) based in part on an *in silico* prediction pipeline established by AU. Following the synthesis of the oligonucleotides (RBT) they were validated extensively (AU, MPI) by

(i) toxicity assays to filter out siRNAs that lead to cell death,

(ii) *in vitro* knockdown assays to determine if the individual siRNA/antisense molecule leads to knockdown of the intended gene,

(iii) interferon induction assays to recognize potential interferon inducing effects and

(iv) influenza virus replication assays to determine if knockdown of particular genes can indeed reduce the replication rate.

Ferret-specific siRNAs were validated with commercially available primary ferret cells, since no ferret cell line was available. However, initial studies revealed that these primary cells grew very slowly and were difficult to transfect with siRNAs. For these reasons the ferret model was followed up. Validation of porcine siRNAs was carried out at AU using an *in vitro* model of primary porcine polarized airway epithelial cells (PAEC) grown on permeable membranes in a liquid/air interface to enable screening for improved delivery to ciliated and mucus-producing primary cells. Improvement of siRNA delivery was addressed by different strategies such as (1) mucopenetrative nanoparticels with high molecular PEI modified by NAC or with 0.8 kDa PEI, and (2) mucoadhesive chitosan as well as (3) mucolytic papain-conjugated nanoparticles. Papain enzyme is a mucolytic enzyme which can act on the mucus layer and reduce the viscosity. It acts on the non-glycosylated domain which is protease sensitive and supports the penetration of the mucus layer. Exposure of papain on the surface of a particle might, therefore, be advantageous for the delivery of therapeutic oligos to the lung. The design of papain/ssPEI nanoparticles is shown in Figure 15.

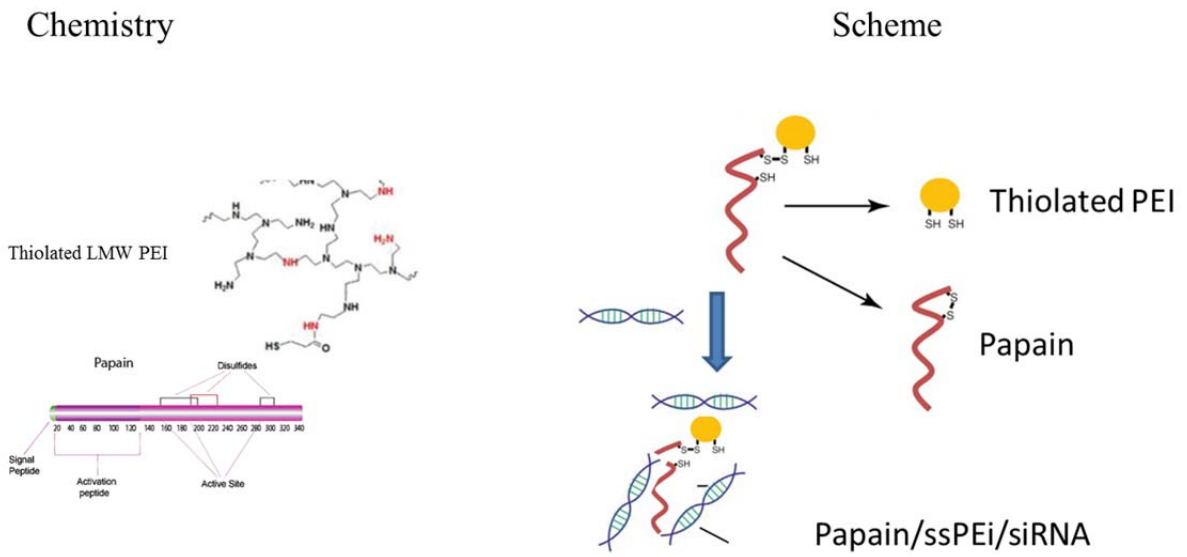


Fig. 15 Design of papain\ssPEI nanoparticles. *The papain enzyme was conjugated with thiolated polyethylenimine which, due to its positive charge, readily complexes with the negatively charge nucleotides to form a nanoparticle.*

Although papain binds to the thiolated PEI through a thiol-thiol bond, we confirmed full activity of the enzyme after nanoparticle formation. Papain is a protease and has to degrade the protein matrix in the assay. Enzymatic activity of papain in the nanoparticle form was confirmed via its ability to degrade the proteins in the assay. Presence of papain with the thiolated PEI was confirmed with NMR and the particle morphology was observed with transmission electron microscopy. The novel papain-conjugated nanoparticle system exhibited promising uptake and delivery efficiency in mucus-producing cell lines *in vitro*. However, *ex vivo* delivery studies with trachea and alveolar tissue revealed that none of them entered cells efficiently.

In the course of the project antisense DNA oligonucleotides flanked by locked nucleic acids, so called gapmers, were also included in the studies and showed potent gene knockdown without the need for a transfection agent, compared to siRNA, which requires an assisting delivery system. In addition, they generally showed a stronger degree of knockdown compared to siRNAs. Based on the *in vitro* validation studies, one lead antisense oligonucleotide against the ATPase gene (AS-ATP6V0D1, Fig.16) - with and without palmitoyl or glycin modification in the flanking regions - was selected for subsequent *in vivo* knockdown studies since they consistently revealed efficient knockdown, low cytotoxicity and strong inhibition of viral replication.

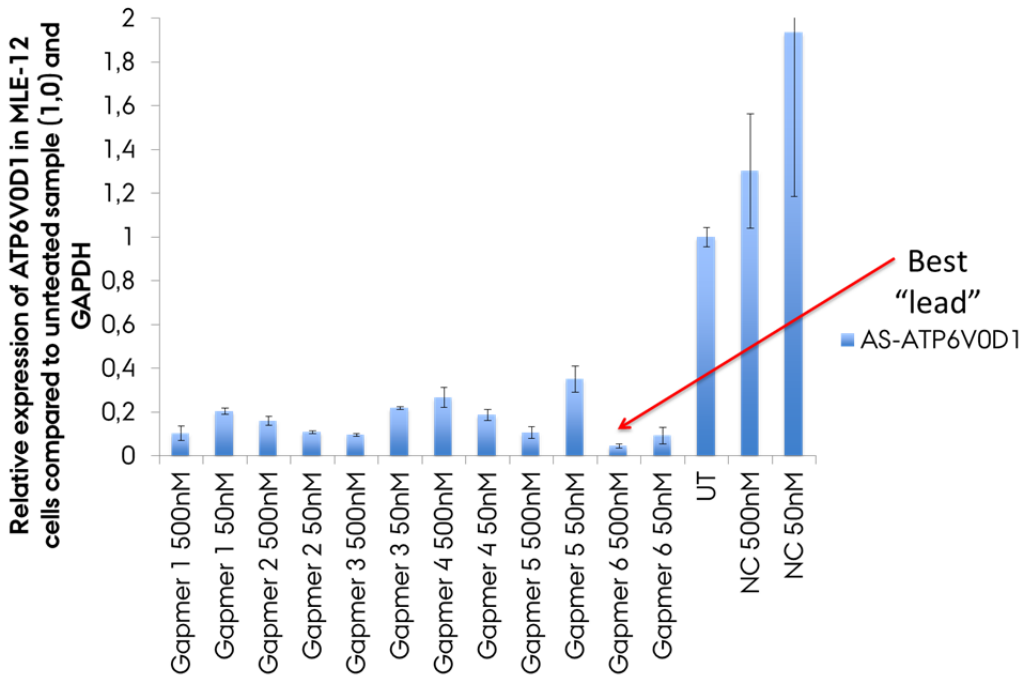


Fig. 16 ***The knockdown efficiencies of validated gapmers.*** *The gene expression of ATPase gene and the capacity of gapmers with transfection agent Hiperfect to induce knockdown was determined by performing qRT-PCRs. All results were normalized based on the gene expression level of a reference gene GAPDH (UT). The scrambled gapmer sequence (NC) was used as a negative control.*

Delivery optimisation and in vivo validation of lead siRNAs (WP6)

Delivery of siRNA to lung epithelial cells was a major challenge and subject to intensive studies in the ANTIFLU project. The initial approach was to encapsulate *in vitro*-validated siRNA into nanoparticles using a range of different polymers, then test the delivery of siRNA to the lungs, bronchi or trachea in the mouse model. Although promising results were obtained *in vitro* using physiologically relevant model systems, the *in vivo* results were less encouraging. Extended lung deposition was observed using chitosan/siRNA nanoformulations, however there was very little translocation of siRNA into epithelial cells. To continue with the attempt to deliver siRNA lead compounds, AU developed a novel mucopenetrative papain-conjugated nanoparticle system that exhibited promising uptake and delivery efficiency in mucus-producing cell lines *in vitro*. However, this formulation showed indications of toxicity *in vivo* and no observable uptake was detected for the papain-conjugated nanoparticle systems *in vivo*.

During the ANTIFLU project an alternative nucleic acid therapeutic, antisense DNA oligonucleotides flanked by locked nucleic acids (gapmers), showed potent gene knockdown without the need for a transfection agent (gymnotic delivery), in contrast to siRNAs, which require an assisted delivery system. Based on this observation, the consortium turned its focus to the validation of primary hit antisense oligonucleotides, in addition to already established siRNA leads, by using an appropriate *in vivo* mouse model to deliver the drugs into the lungs. To further improve pharmacokinetic behavior (retention time, mucus penetration, epithelial cell uptake and circulation,) chemically modified oligonucleotides with e.g. amino-LNA, galactyl or palmitoyl groups were tested. The palmitoyl-conjugated gapmer was found to be functional and non-toxic *in vitro*. Based on this, unmodified and palmitoyl-modified gapmers were expected to enter the mouse lung epithelial cells when administered intratracheally and hence were the most promising inhibitors to continue with. Finally three gapmers targeting vATPase (NAC 7622, NAC 7626, NAC7489) were selected for *in vivo* knockdown studies. For this purpose a new method for intratracheal application (miniaturized bronchoscopy) was used in

cooperation with Charité University Hospital Berlin. The experimental setup was further optimised by enrichment of murine lung epithelial cells from whole lung tissue by FACS sorting, to determine the knockdown rate in only those cells that represent the target cells for influenza infection. The knockdown studies using vATPase-specific naked gapmers resulted in a 50% reduction of gene expression in epithelial cells of the murine lung (Fig. 17). Thus an important milestone (MS12, Effective pulmonary delivery in mice gene silencing in vivo) of the RNAi part of the ANTIFLU project was achieved and provided the basis for subsequent infection studies *in vivo*.

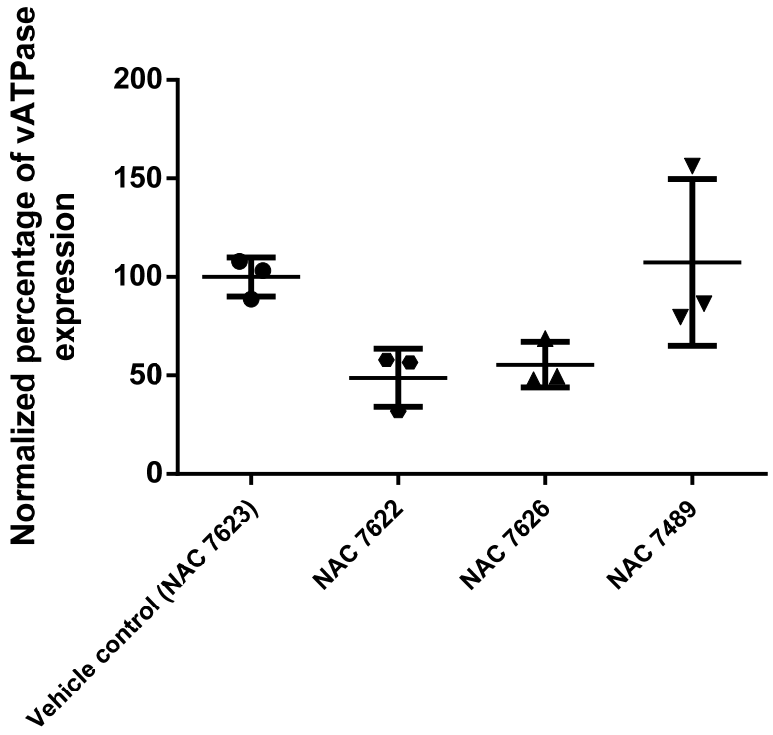


Fig. 17 ***Knockdown efficacies of three vATPase specific gapmers compared to a scrambled control.*** *Gapmers were applied intratracheally by bronchoscopy on days 0 and 2 and the level of knockdown in lung epithelial cells was determined on day 3 by quantitative RT-PCR.*

In a subsequent mouse experiment, the intratracheal application of the vATPase-specific gapmers 2 days and 4 hours before infection with influenza virus unfortunately did not lead to a reduction in the virus load in the lungs of the mice. The most likely reason for this might be the relatively low *in vivo* knockdown efficacy of 50%, which seemed insufficient to block influenza replication. In comparison, *in vitro* knockdown efficiency of selected siRNAs and gapmers was ≥ 70%. Concluding further efforts to increase the knockdown efficiency *in vivo* are required to finally evaluate the potential of this approach for therapy of influenza infection *in vivo*.

In summary, in vitro studies revealed unmodified and palmitoyl-conjugated gapmers against vATPase as potent inhibitors of vATPase expression and, as an important result, intratracheal application of specific gapmers to mice led to a 50% knockdown of vATPase expression in lung epithelial cells. Parts of the delivery studies were included in two publications: 1. Mucin-mediated nanocarrier disassembly for triggered uptake of oligonucleotides as a delivery strategy for the potential treatment of mucosal tumours (Howard K, 2015, Nanoscale in press) and 2. Nucleic Acid Nanotechnology (Kjems J., 2014, Nucleic Acids and Molecular Biology, Vol. 29).

Partners mainly involved (and their main task):

AU (design, formulation and validation of nucleotide-based inhibitors *in vitro, in vivo* knockdown studies)

RBT (design and synthesis of nucleotide-based inhibitors)

MPI (*in vitro* validation of knockdown, antiviral activity, cytotoxicity, interferon induction and *in vivo* knockdown and efficacy studies)

IC (delivery of ferret sequences and a primary ferret cell system)

Summary

In the beginning of the ANTIFLU project the plan was to develop a clinical candidate within one of the three approaches (kinase inhibitor, non-kinase inhibitor, nucleotide inhibitor), which was a very ambitious goal in the light that drug development is generally known to exceed a decade of time and include several levels of possible pitfalls and failure. Even though the ultimate goal in this regards was not achieved, by the end of the project, all three approaches have been pursued towards respectable stages, owing to the high level and interdisciplinary expertise as well as the excellent cooperation of the consortium partners.

- In the kinase part of the ANTIFLU project a highly promising CLK1 lead candidate was developed.
- In the non-kinase part of the ANTIFLU project a vATPase lead was successfully nominated and further improved regarding safety margin, metabolic stability and half-life by MedChem. In addition, two commercial available inhibitors against the ANTIFLU-prioritized target XPO1 were tested for their suitability as clinical candidates with exceptional outcome and success.
- In the RNAi part of the ANTIFLU project two vATPase-targeting gapmers were shown to induce knockdown of the target gene in lung epithelium of mice upon intra-tracheal application

Accordingly, all milestones and deliverables, with exception of those connected to the lead optimization of a kinase inhibitor and the nomination of a clinical candidate, were completed. Apart of that, substantial amounts of scientific knowledge and know-how were generated within the consortium. Parts of this data will be contributed to the public via publications, manuscripts and an international patent application.

In addition to the partners listed in the description above, the continuous active advisory support of IP related to influenza research as well as public health issues should be noted here, together with consultancy of MT with regard to *in vivo* toxicity studies. ART supported the coordinator in all aspects of project management, in particular the financial management of the ANTIFLU project.

4. Potential impact, main dissemination activities and exploitation of results

4.1 Socio-economic impact

ANTIFLU had an overall positive impact on employment and workforce distribution in the participating organisations (academic and research organisations and innovative SMEs). Over the 5.5 years of the project, more than 94 people (53 women and 41 men) have contributed at some point to the project. They provided their expertise to the different work packages and were involved in outreach and education activities, including e.g. through student visits and interactions with schools pupils.

The ANTIFLU public workshop in November 2016 served to present the project approach and strategies for generating new effective host directed anti-influenza drugs and more generally to raise awareness about host directed therapy (see Section 4.3 below).

ANTIFLU did not directly work with or target policy makers. However, results originating from the project could be used by policy makers in the relevant fields, especially in the areas of research and innovation and public health. Especially, the implications of the project results regarding alternative therapy approaches by targeting human host cell factors might be relevant for the set up of future funding programs by the national and international funding agencies.

Due to the required confidentially about the targets and inhibitors involved in this drug development project only one publication in a peer-reviewed journal and two book chapters have been published during the project (see Table 2.1.1). Importantly, in 2015 a patent about the synthesis, structure, antiviral and anticancer activity of a certain family of CLK1 inhibitors was filed (see Table 2.2.1). Additional dissemination activities are described in Section 4.3 and listed in Table 2.1.2.

4.2 Wider societal implications of the project

The long lasting intention of the ANTIFLU project was to develop an alternative, new strategy for influenza treatment which is less susceptible for resistance development of the virus and is effective against a board spectrum of influenza virus strains. By pursuing a host-directed therapy approach the consortium could demonstrate the potential of this new strategy to overcome the above mentioned limitations of resistance and viral variability. Even though the project finally did not result in a candidate for a clinical phase I study the gained knowledge which will in part be published in 2017 provide good evidence for the benefit and feasibility of the host directed therapy for influenza treatment.

Apart from that the close cooperation of research groups, SMEs and clinical partners within the consortium turned out to allow high quality translational research which led to respectable results within the given project duration of 5.5 years and a mutual benefit of the partners.

4.3 Main dissemination activities

A dissemination highlight was the ANTIFLU public workshop which was held on 17 November 2015 at the Harnack House of the Max Planck Society in Berlin (see D8.4). Through this public outreach meeting, the ANTIFLU Consortium presented with several keynotes its approach and strategies for generating new effective host directed anti-influenza drugs and raised awareness about host directed therapy.

In addition, the ANTIFLU project and its results have been presented at several national and international conferences (see Section 2.1.2). Examples of events at which the project was presented include the National Symposium on Zoonoses Research (Berlin, Germany; 2011), the European Science Foundation Conference on “Antiviral RNAi: From Molecular Biology Towards Applications” (Pultusk, Poland; 2012), the Influenza Research and Development Conference and the 9th Anti-Infectives Partnering & Deal-Making (San Francisco, USA; 2012), the 3rd and 4th International Influenza Meeting (Münster, Germany; 2012 and 2014), the European Congress of Virology (Lyon, France; 2013)the Summer School in Nanomedicine and Innovation (Tel Aviv University, Israel; 2014);

the 24th Annual Meeting of the Society For Virology (Alpbach, Austria; 2014), the Annual Meeting of the Danish Society for Pharmacology, Toxicology and Medicinal Chemistry (DSFTM) (Sandbjerg Gods, Denmark; 2015) and the 13th P4EU (Protein Production and Purification Partnership in Europe) meeting (Rehovot, Israel; 2016).

Due to the required confidentiality about the targets and developed inhibitors most of the results could not yet been published. Up to now one publication in a peer-reviewed journal and two book chapters are published.

Nanoscale

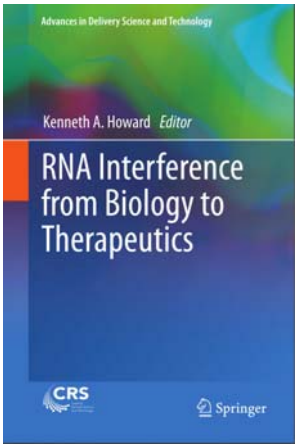
PAPER View Article Online View Journal

CrossMark

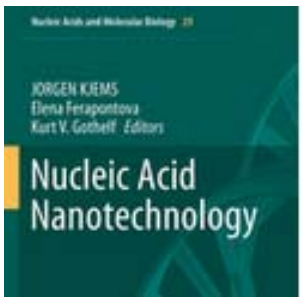
Cite this: DOI: 10.1039/c5nr07206a

Mucin-mediated nanocarrier disassembly for triggered uptake of oligonucleotides as a delivery strategy for the potential treatment of mucosal tumours†

A. Martirosyan,[a] M. J. Olesen,[a] R. A. Fenton,[b] J. Kjems[a] and K. A. Howard*[a]



12 **Genome-Wide RNAi Screening to Identify Human Host Factors Crucial for Influenza Virus Replication** 243
Katharina Ahrens and Alexander Karlas



Jørgen Kjems , Nucleic Acids and Molecular Biology, Vol. 29 (2014)

The following two manuscripts about CLK1 are prepared (see D3.6 and D3.7) and will be submitted in 2017.

Expression Purification and Crystallization of Clk1 Kinase – a possible target for anti influenza therapy

Noa Dekel, Yael Einsenberg-Domovich, Alexander Karlas, Jens Peter von Kries, Mario Lebendiker, Tsafi Danieli, and Oded Livnah,

Submission is planned to the journal “Protein Expression and Purification” journal

Abstract:

CLK1 is a dual specificity kinase capable of autophosphorylation on tyrosine residues and on Ser/Thr on its substrates. Recent studies by members of our group and by others have demonstrated that CLK1 has an important role in the replication, and more specifically in the alternative splicing of viruses such as Influenza A and chikungunya. We have previously shown that inhibition of CLK1 leads to decrease in viral budding, rendering the protein a favorable target for anti-viral inhibitors. In this paper we describe our attempts and detailed procedures to obtain recombinant kinase domain of CLK1 in suitable amounts for crystallization in complex with specific inhibitors. The key solution for reproducibility of crystals is in devising and refining the expression and purification protocols leading to homogeneous protein preparation. Co-expression of CLK1 with λ-phosphatase and careful purification has yielded crystals of CLK1 complexed with the KH-CB19 inhibitor that diffracted to 1.65 Å high resolution. These results pave the path to screening of more structures of CLK1 complexed compounds leading to further optimization of their inhibitory activity. Moreover,, since kinases are desired targets in numerous pathologies, the approach we developed in this study: co-expression of kinases with λ-phosphatase can be adopted as a general protocol in numerous kinase targets for obtaining non-phosphorylated (inactive) forms, suitable for biochemical and structural studies and facilitate the development of novel inhibitors.

Regulation of influenza A virus mRNA splicing by CLK1

Anita Artarini, Michael Meyer, Yujin Shin, Kilian Huber, Nikolaus Hilz, Franz Bracher, Daniel Eros, Lazlo Orfi, Georgy Keri, Sigrid Goedert, Rike Zietlow, Jens von Kries, Tsafi Danieli, Oded Livnah, Olivier Moncorgé, Rebecca Cocking, Wendy Barclay, Thomas F. Meyer and Alexander Karlas

Abstract:

Influenza A virus carries eight negative single-stranded RNAs that together encode 14 viral proteins, three of which are produced from splicing process. Several genome-wide screens performed to identify essential host factors for influenza A virus replication revealed a necessity for splicing and splicing-related factors in influenza infection, including Cdc-like kinase 1 (CLK1). This CLK family kinase is known for its role in alternative splicing regulation through phosphorylation of serine-arginine rich (SR) proteins. To examine the influence modulation of splicing regulation has on influenza infection, we analyzed the effect of CLK1 knockdown and inhibition. We found that CLK1 knockdown led to a reduction of influenza A/WSN/33 virus replication and an increase of the splicing rate of segment 7 mRNA in A549 cells. Infection of CLK1-/- mice with influenza A/England/195/2009 virus resulted in reduction of virus replication. Screening of newly-developed CLK inhibitors revealed several compounds that can effectively inhibit influenza A/WSN/33 virus replication in A549 cells. Treatment of A549 cells with an inhibitor that shows higher specificity towards CLK1 had a similar effect on influenza mRNA splicing regulation, as did knockdown of SRSF3. Taken together, our findings indicate that targeting influenza splicing regulation may represent a novel therapeutic approach.

In 2015 a European patent about the chemical structure and the antiviral and anticancer activity of a CLK1 inhibitor family was filed which resulted in an international patent application in 2016.

ANTIFLU has a dedicated website (www.antiflu-project.eu) to present the project and advertise project events (such as the ANTIFLU public workshop). Here the public part of the final report will be deposited upon approval by the European Commission.

4.4 Exploitation of results

The screening for potent CLK1 specific inhibitors and the validation and optimisation of selected hits resulted in a European patent application of VCR and MPI in February 2015:

EP3056202: **Benzopyrolidone derivatives possessing antiviral and anticancer properties**

The invention relates to benzopyrrolidone derivatives and/or pharmaceutically acceptable salts thereof for use in the treatment of infection diseases, and pharmaceutical composition containing at least one of the said benzopyrrolidone derivatives and/or pharmaceutically acceptable salt thereof.

Based on this application in February 2016 an international patent application was filed:

WO 2016/131789 A1: **Benzopyrolidone derivatives possessing antiviral and anticancer properties)**

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 August 2016 (25.08.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/131789 A1

(51) International Patent Classification:
A61K 31/404 (2006.01) *C07D 209/20* (2006.01)
A61K 31/4178 (2006.01) *A61P 35/00* (2006.01)
A61K 31/439 (2006.01) *A61P 31/12* (2006.01)
A61K 31/4439 (2006.01) *C07D 403/12* (2006.01)
A61K 31/454 (2006.01) *C07D 401/14* (2006.01)
A61K 31/496 (2006.01) *C07D 401/06* (2006.01)
A61K 31/5377 (2006.01) *C07D 401/12* (2006.01)
C07D 453/00 (2006.01)

(21) International Application Number:
PCT/EP2016/053204

(22) International Filing Date:
15 February 2016 (15.02.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15155298.1 16 February 2015 (16.02.2015) EP

(71) Applicants: **MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.** [DE/DE]; Hofgartenstrasse 8, 80539 Munich (DE). **VICHEM CHEMIE LTD.** [HU/HU]; Herman Ottó u. 15., 1022 Budapest (HU).

(72) Inventors: **KÉRI, György**; Modori u. 4, 1021 Budapest (HU). **ÖRFI, László**; Batthyány u. 92., 1161 Budapest (HU). **HORVÁTH, Zoltán**; Vörösvári út. 11. 9/49, 1035 Budapest (HU). **SZOKOL, Bálint**; Herman Otto u. 15., 3, epulet VICHEM, Budapest 1022 (HU). **DOBOS, Judit**; Lajos u. 53-63/C I/1, 1036 Budapest (HU). **NEMES, Zoltán**; Diószeghy Sámuel utca 15 7/92, 1089 Budapest (HU). **SZÁNTAI KIS, Csaba**; Viola u. 34/A 3/3, 1094 Budapest (HU). **ERŐS, Dánilel**; Csantavér u. 3 fsz..2, 1146 Budapest (HU). **BREZA, Nóra**; Szinbád u. 3. 14/15D, 1039 Budapest (HU). **BASKA, Ferenc**; Rózsa u. 59. fsz.6., 1064 Budapest (HU). **KARLAS, Alexander**; Kopernikusstr. 18a, 10245 Berlin (DE). **GÖDERT, Sigrid**; Emsstr. 32, 14612 Falkensee (DE). **MEYER, Thomas F.**; Haydnallee 21, 14612 Falkensee (DE).

(74) Agent: **ARTH, Hans-Lothar**; ABK Patent Attorneys, Jasminweg 9, 14052 Berlin (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,

[Continued on next page]

(54) Title: BENZOPYRROLIDONE DERIVATIVES POSSESSING ANTIVIRAL AND ANTICANCER PROPERTIES

Apart from this patent application and the publications listed in Table 2.1.1 the consortium generated comprehensive scientific knowledge and know how within the three scientific branches of the project (kinase, non-kinase, and RNAi). The most important ones for exploitation are summarised in table 2.2.2. and described here:

In the first months of the project a "**filter rank-select system**" was developed to identify the most potent hits out of a primary hit list resulting from the genome wide influenza screen for the subsequent development of small molecule inhibitors or adequate siRNA inhibitors. For this extensive selection process own data, relevant literature data and bioinformatics analyses were integrated and ranked. This method can be applied to other hit list and might be part of a future publication about the vATPase lead development.

The **knowhow generated in the frame of the vATPase lead development and optimization** including the development of different assays systems for screening and validation as well as SAR identification, resistance studies and in vivo studies can be used for the development of inhibitors against related kinases and are planned to be summarized and published in a peer-reviewed journal.

Provided that further in vivo efficacy studies will lead to positive in vivo PoC data a **compound patent about the optimized vATPase lead** including its structure and data about its antiviral activity in vitro and in vivo will be filed.

The same is true for the **lead gapmers specific to vATPase**. Here first the in vivo knockdown efficiency needs to be improved by optimised application protocols. Provided that the increased knockdown will inhibit the viral load in infected mice significantly the gapmers would be protected by a patent application and the results would be published in a peer-reviewed journal.

4.5 ANTIFLU partners

The ANTIFLU consortium is composed of the following organisations:

- Max Planck Institute for Infection Biology (MPI)
- Vichem Chemie Research Ltd. (VCR)
- Aarhus University (AU)
- Lead Discovery Center GmbH (LDC)
- Institut Pasteur (IP)
- ARTTIC SA (ART)
- MediTox S.R.O. (BT)
- Imperial College London (IC)
- Ribotask (RBT)
- The Hebrew University of Jerusalem (HUJI)
- Leibnitz Institute for Molecular Pharmacology (FMP)

Contact details:

Prof. Thomas F. Meyer

Max Planck Institute for Infection Biology (MPI)
Charitéplatz 1
10117 Berlin
Germany

Phone: +49 30 28 460 402
E-mail: meyer@mpiib-berlin.mpg.de

ANTIFLU public website: http://www.ANTIFLU-project.eu/