Biomek FX^P Laboratory Automation Workstation as a tool for discovering targets & compounds for host-directed influenza therapy

Abstract

Influenza viruses (IVs) represent a major public health burden. As this class of viruses depends on host factors to complete their life cycle, which takes place inside human cells, these cellular components present attractive anti-IV drug targets. To systematically identify the most promising factors for such a host-directed therapy, we screened a siRNA library for cellular genes involved in viral multiplication. Upon identification of relevant genes, we screened chemical inhibitors of the target genes for anti-viral activity. To achieve the high throughput required for these screening campaigns, we employed the Biomek FX^P Laboratory Automation Workstation enclosed in a BSL-2 or -3 safety cabinet. Using this strategy, we identified several agents that inhibit IVs in the nano-molar range without cytotoxicity.

Introduction

IVs are among the pathogens with the highest clinical relevance, as they are responsible for approx. 250,000 to 500,000 deaths per year. Because resistance to vaccination and existing anti-virals develops rapidly, there is an urgent need for new drugs against this class of viruses. IVs multiply within human cells and strongly depend on host cell functions to do so. Consequently, host factors represent anti-IV drug targets. To systematically identify the most promising factors for such a host-directed therapy, we screened an arrayed (384-well format) siRNA library for genes involved in IV replication. Four different IV strains (including a highly pathogenic H5N1 strain) were compared to identify genes relevant to a broad range of IV types. The siRNA library was further screened for immunogenic and cytotoxic siRNAs, as these can affect viral replication indirectly. This extensive screening campaign required a high degree of automation. To tackle this challenge, we employed the Biomek FX^P Laboratory Automation Workstation for all pipetting steps, including siRNA transfection, cell plating, virus infection, and different cell-based assays (luciferase activity, cell proliferation, virus titer measurement by fluorescent foci). Using this system, we identified 133 human genes that are required for multiplication of all IV strains tested, and are thus potential targets for a host-directed therapy. Subsequently, we screened for chemical inhibitors of the most promising targets. Again, we used the Biomek FX^P to first prepare serial dilutions, which are subsequently used to record dose-response curves in order to determine the IC_{50} and CC_{50} values of the compounds. Several agents inhibiting IVs in the nano-molar range without cytotoxicity were identified. In essence, the Biomek FX^P was the core tool of our campaign and proved to be suitable for various high throughput cell biology applications.

The system

The central tools employed in our screening campaign were two Biomek FX^P Laboratory Automation Workstations. To maintain sterility and protect the environment from infectious pathogens, the systems – equipped either with two 384 multichannel heads or with the hybrid system containing the 384 multichannel head and the Span-8 pod – were enclosed in custom-made BSL-2 or -3 safety cabinets. The Biomek FX^{P}s were integrated in environments consisting of an automatic plate incubator (for cultivation of cells), an ambient plate hotel (for room temperature incubation steps) and two automatic multi-dispensers. The BSL-2 system was further connected by a robotic handler to

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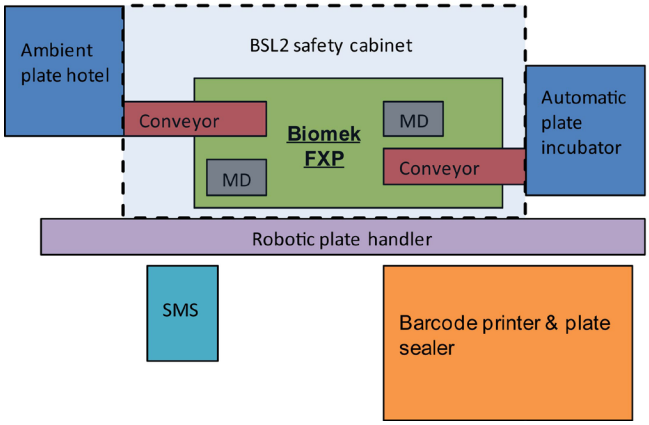
Figure 1: Biomek FXP working environment (BSL2). (A) Schematic of working environment. MD: multi-dispenser, SMS: scanning multi-well spectrophotometer. (B) Photograph of working environment.

A



B

the scanning multi-well spectrophotometer EnVision® Multilabel Reader (Perkin Elmer), as well as an automatic plate sealer and an automatic barcode label printer. It was used for (1) siRNA handling, (2) the interferon induction siRNA screen and (3) cytotoxicity testing (siRNA, small molecule) and is depicted in Fig. 1. The BSL-3 system was employed for virus replication screens (siRNA, small molecule).

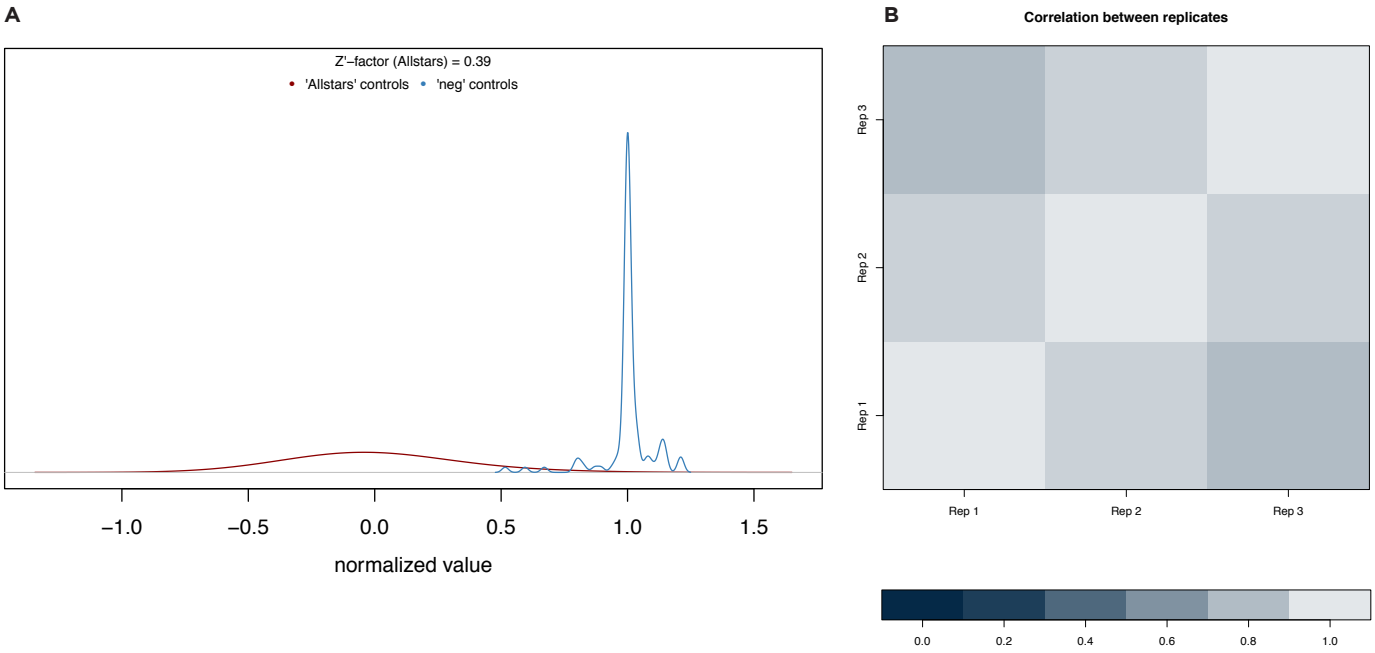
The siRNA screen

The library analyzed consisted of 3,482 siRNAs (targeting 1,208 cellular genes) distributed on thirteen 384-well plates. The siRNAs were dissolved, titrated, and distributed on ready-to-use plates using the Biomek FX^P. Plates were automatically labeled with barcodes and sealed using the automatic label printer and sealer, respectively, and manually transferred to -80°C freezers. Information about plates and barcodes was stored in a laboratory information management system.

The screen was performed in A549 human lung epithelial cells, and the siRNA library was independently screened for three parameters: interferon induction, cytotoxicity, and effect on viral replication. The induction of interferon was assayed because siRNAs can potentially induce an interferon response independently of the target gene, and thereby impair viral replication indirectly (Echeverri and Perrimon, 2006). To monitor interferon induction, genetically modified A549 cells expressing luciferase under control of an interferon sensitive promoter were employed. Read-out (i.e. luciferase activity) recording was conducted 24 h post-transfection (p.t.), using the EnVision Multilabel Reader. Cytotoxic siRNAs will impair viral replication even when the target gene is involved only in cell viability and not in viral replication itself. Therefore, we also screened for cytotoxic siRNAs using a colorimetric, tetrazolium salt-based assay (Berridge et al., 2005). Read-out of this test was conducted at 48 h p.t., also using the EnVision Multilabel Reader. The parameter of highest interest, i.e. permissiveness of cells for IV replication, was investigated at 48 h p.t by infecting cells and determining production of virus progeny at appropriate time points post-infection (36 or 96 h, using a fluorescent focus assay (Karlas et al., 2010). To this end, the supernatant of infected A549 cells was transferred onto uninfected cells, which were fixed seven hours later. Subsequently, the cells were manually stained for viral antigen and examined by automated microscopy and image segmentation analysis. This screen was conducted with all four IV strains.

As the central tool, the Biomek FX^P was used for plate handling, washing of cells, and liquid dispensing. Some liquid dispensing steps (e.g. addition of luciferase substrate or tetrazolium salt) were carried out using the multi-dispensers. The microtiter plates were shuttled between the Biomek FX^P and the automatic plate incubator, as well as the ambient plate hotel via conveyors. Barcode scanners at the conveyors allowed automatic documentation of the plate handling process as the microtiter

Figure 2: Quality control during siRNA screening. (A) Data of a representative siRNA screening round for virus replication. Red curve indicates a non-targeting siRNA, blue curve indicates an siRNA targeting the viral genome. (B) Spearman correlation analysis for three replicate experiments (virus replication) of a representative plate.



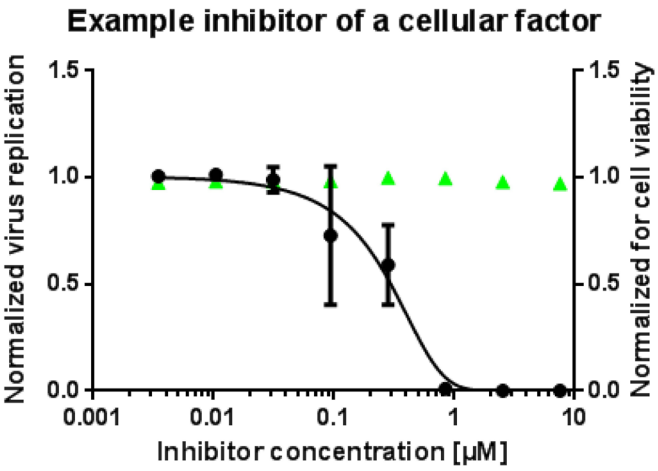
plates carried barcodes. Transfer of plates between the Biomek FX^P and the EnVision Multilabel Reader was performed using the robotic handler (Fig. 1A). The Biomek FX^P scheduling software allowed convolution of different robotic protocols, in order to achieve optimal efficiency of the system.

Data analysis was performed using the cellHTS2 package for R web based analysis package (Boutros et al., 2006). The Spearman correlation coefficient and the Z' (i.e., difference between positive and negative control) were employed as quality metrics. Effects of positive and negative controls were easily distinguished by Z' values above 0.3 (Fig. 2A). Also reproducibility between replicate experiments was high with correlation coefficients of around 0.6 - 0.8 (Fig. 2B). Immunogenic and cytotoxic siRNAs were removed from analysis of viral replication screens. Overall, we identified 133 cellular genes that were essential for replication of all four IV strains tested. These 133 genes represent potential targets for a host-directed therapy for IV infection.

The small molecule screen

We selected several of the 133 genes for the development of small molecules targeting the respective gene products (i.e. proteins). Together with collaborating medicinal chemists, we developed several substance series that were screened for anti-viral activity and cytotoxicity, employing the assays described above. Using the screening facility within the BSL-3 lab we were able to setup a Biomek FX^P protocol that allows (1) compound dilutions, (2) pre-treatment of cells with up to 96 compounds at eight different concentrations, (3) IV infection of A549 cells in 384-well plates, (4) re-addition of compounds using the same range of concentrations, and (5) transfer of virus-containing supernatants to uninfected cells for virus titer quantification as described above. In this way, we successfully identified several small molecules that inhibit IV replication in the nano-molar range without cytotoxicity. Fig. 3 depicts the dose-response curves for anti-viral activity and cytotoxicity of one of the compounds.

Figure 3: Inhibitor of a cellular factor inhibits viral replication without cytotoxicity. A549 cells were infected with influenza virus in the presence of a small molecule inhibitor targeting a hit gene identified in the siRNA screen. Infectious virus progeny (black) and cell viability (green) were determined 48 h post-infection by fluorescent focus assay and tetrazolium salt-based cell viability assay, respectively.



Conclusion

We performed an extensive siRNA screen to identify targets for a host-directed therapy of influenza virus infections. Small molecule inhibitors against selected targets were developed and screened for anti-viral activity and cytotoxicity. Several non-cytotoxic inhibitors of cellular factors with high anti-viral activity were identified. Throughout all screens, the Biomek FX^P Laboratory Automation Workstation was the essential tool for our campaign and proved to be suitable for various cell biological high throughput cell biology applications.

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