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Review

New-generation screening assays for the detection of anti-influenza compounds targeting viral and host functions

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Current options for influenza antiviral therapy are limited to the neuraminidase inhibitors, and knowledge that high levels of oseltamivir resistance have been seen among previously circulating H1N1 viruses increases the urgency to find new influenza therapeutics. To feed this pipeline, assays that are appropriate for use in high-throughput screens are being developed and are discussed in this review. Particular emphasis is placed on cell-based assays that capture both inhibitors of viral functions as well as the host functions that facilitate optimal influenza virus replication. Success in this area has been fueled by a greater understanding of the genome structure of influenza viruses and the ability to generate replication-competent recombinant viruses that carry a reporter gene, allowing for easy monitoring of viral infection in a high-throughput setting. This article forms part of a symposium in *Antiviral Research* on "Treatment of influenza: targeting the virus or the host."

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1. Introduction

The use of high-throughput screening (HTS) technology for antiviral discovery is a fairly recent endeavor, first undertaken exclusively by the pharmaceutical industry and now also performed by academic scientists. The development of HTS has been driven by increasing advances in automation and the ability to handle large datasets. It has also expanded the types of target that can be explored and consequently assay development, particularly of cell-based assays, is a major part of all antiviral HTS campaigns.

As a small RNA virus, influenza virus encodes a limited number of proteins and thus there are only a few viral functions that are considered to be tractable drug targets by traditional standards. This essentially means that the target must have a function that is amenable to inhibition by a small molecule. The current two classes of approved antivirals for influenza target either the ion channel function of the M2 protein or the neuraminidase function of the NA protein. The neuraminidase inhibitors (NAI) were developed through the rational design of small molecules that mimic sialic acid and bind with high affinity to the active site of NA (Gubareva et al., 2000). The adamantanes are an interesting example of an antiviral whose approval preceded knowledge of the target (M2) or the function of the target as an ion channel. Moreover, the precise mechanism of action is still under debate following publication of structures showing different placement of the drug relative to M2 (Cady and Hong, 2008; Cady et al., 2010; Pielak and Chou, 2010; Stouffer et al., 2008). Other well-characterized viral functions that should be druggable are the RNA-dependent RNA polymerase activity of PB1 and the endonuclease function of PA. Apart from the fact that the description of PA endonuclease activity was only made in 2009 (Dias et al., 2009; Yuan et al., 2009), the major reason that these targets have not been explored fully is the inability to produce purified, full-length and active polymerase proteins, which severely limits the development of biochemical screening assays.

The examples above refer to viral functions that are considered to be validated targets, as it is known *a priori* that they are essential for influenza virus growth, and biochemical assays can (or could) be developed to screen for specific inhibitors of that function. Alternatively, one can cast a wider net by not requiring knowledge of the target or function upfront and instead using a phenotypic readout such as virus replication. This approach requires a cell-based assay and it is in this area that we have seen most development in the influenza virus HTS field. The advantages are: (i) that it potentially allows one to capture all stages of the virus life-cycle in one assay, (ii) it detects inhibitors of cellular functions that are required for virus replication, and (iii) it may reveal unknown functions of viral proteins that are susceptible to small molecule inhibition. This review will focus on the new tools that have been developed for influenza antiviral drug discovery, with an emphasis on the use of fluorescent or luminescent reporters and the development of novel cell-based assays.

2. Suitable HTS assays for influenza antiviral discovery

The type of assay chosen for a screen depends on the question being asked and what tools are available. If the purpose is to identify inhibitors of as many different steps of the influenza virus life-cycle as possible, then an assay involving virus infection of cells must be used, preferably under conditions of multi-cycle replication (see Section 3.1). The readout for this type of assay can vary from antibody-based detection of viral proteins, to expression of reporter genes encoded by the virus (see Section 3.2), to indirect measurements such as cytopathic effect (see Section 3.4). Cell-based assays with reporter readouts can also be used to assess specific stages of the virus life-cycle (e.g. entry or replication phases, see Section 3.3), whereas if the purpose of the screen is to find inhibitors of a specific protein it is preferable to analyze this target in isolation using a biochemical assay that provides a readout of the protein function (see Section 4.1). In cases where a crystal structure of the protein target is available it may be possible to use an *in silico* approach where large libraries of small molecules are computationally docked onto the structure to identify those with potential binding properties (see Section 4.2). These predicted hits can then be validated in a functional assay, either biochemical or cell-based. The design of such assays obviously requires extensive prior knowledge of the functional properties of the protein target and of how this property affects virus replication, as well as the availability of appropriate tools e.g. purified protein. In many cases this information or the tools (or both) are lacking and increasingly antiviral screens are being conducted using cell-based assays without any knowledge of a specified target. Rather, the objective is to identify small molecules that have an overall phenotypic effect on virus replication and to then employ secondary assays to characterize the mechanism of action and identify the target protein. Increased accessibility to the required automation and to small molecule libraries for those outside the pharmaceutical industry has facilitated the design of new tools for use in cell-based virus assays for HTS.

A successful HTS assay must be robust, have an easy and quantifiable readout and be amenable to miniaturization and the use of robotic machinery. At a very minimum, the assay should function in 96-well format but in most cases further miniaturization to 384-well format is required for compatibility with library plates and pin tools. The smaller, 1536-well format is sometimes used, but it can be more challenging to maintain the assay quality in this format, especially with cell-based assays. The advantage of the smaller format is speed (more compounds screened per day) and reduced costs due to the lower volumes, which can be an important factor if an expensive reagent is required. The assay must be highly reproducible with a large window between the positive and negative controls. A statistical measurement of this is provided by the Z'-factor ($Z' = 1-3(STD_{pos} + STD_{neg})/(MEAN_{pos}-MEAN_{neg})$) and a robust assay that is suitable for use in a screen should have a Z'-factor >0.5 (Zhang et al., 1999). To achieve this, the number of manipulations during the course of the assay should be minimized and it is a common rule that nothing is ever removed from the plate, only added, which helps to reduce variability. If available, reference compounds with known mechanisms of action should be examined in the assay to ascertain assay sensitivity and one should also be aware of possible false positives that may arise from the screen. Another factor to consider is DMSO compatibility as the library compounds will be delivered in 100% DMSO. In general the assay should be able to withstand a range of 0.1–1% DMSO. Finally, in an optimal assay the distribution of signal across the plate will be even with no evidence of edge effects (often due to evaporation from the outside wells) or drifting signal from left-to-right or top-to-bottom.

3. Cell-based assays for measuring influenza virus infection

3.1. Single versus multi-cycle viral replication assays

When designing an assay to monitor influenza virus replication it is important to understand the concept of single cycle vs. multi-cycle replication as this affects the stages of the virus life-cycle that can be captured by the assay. In a single cycle assay, 100% of cells are infected in the first round and thus this type of assay is performed with a high multiplicity of infection (MOI). If the assay readout is viral gene expression, this assay will capture all steps

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