Assessment of High-Throughput Screening (HTS) Methods for High-Consequence Pathogens

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Abstract

Currently, there are no Food and Drug Administration (FDA)-approved antiviral drugs or therapeutics for many of the biosafety level three (BSL-3) and four (BSL-4) pathogens. Many of these high-consequence pathogens, including Venezuelan equine encephalitis virus (VEEV), Ebola virus (EBOV), Marburg virus (MARV), and Lassa virus (LASV), are classified as biothreat agents and the development of therapeutic treatments for these diseases is an important area of research. In recent years, high-throughput screening (HTS) assays have become an effective and robust tool used for drug and therapeutic discovery. There are several types of HTS methods available, including targeted screening, diversity and high-content screening, and RNA interference (RNÁi). These screens have been used effectively with a number of BSL-2 pathogens, but present unique challenges for the BSL-3/4 pathogens due to the requirement for higher level biocontainment facilities as well as biosurety requirements. Addressing and overcoming these challenges is essential for the proper adaptation of HTS into higher biocontainment facilities. In this article, we will discuss the advantages and disadvantages of each of the aforementioned HTS methods in the context of BSL-3/4 containment.

Introduction

Development of antivirals, antibacterials, and other therapeutic drugs has been the focus of scientific and medical research for decades, particularly with the emergence of pathogens resistant to many broad-spectrum antibiotics. In addition to the emergence of drug-resistant pathogens, there are also a number of pathogens that currently do not have any available therapeutics. Until recently, the most common approach to drug discovery has been the "pathology" approach, or "rational drug design," which requires understanding the pathogenesis of the disease, and then using that understanding to find a drug or inhibitor that specifically blocks or inhibits a particular step in the pathogen's lifecycle. A good example of this approach is the use of the compound azidothymidine (AZT) as a treatment for human immunodeficiency virus (HIV). During HIV infection, the virus must reverse transcribe its RNA genome into a cDNA copy to successfully integrate its genome into the host chromosome. AZT, which was originally developed to target reverse transcription of avian retroviruses that may potentially cause cancer, has been successfully used to inhibit HIV reverse transcription [1]. Many RNA viruses and some bacteria can often develop resistance to drugs or therapeutics due to mutation and divergence, whether from lack of proofreading of the RNA polymerase or genetic error. In some cases, the existing drugs can be structurally modified to overcome pathogen resistance. For example, the macrolide class of antibiotics, used to treat Gram-positive bacterial infections, is constantly being structurally modified by adding or changing side chains to produce new drugs for which these pathogens have no resistance [2]. However, while these modifications may work temporarily, the underlying selective pressure is still present, and resistance often reappears [3].

The number of Food and Drug Administration (FDA)-approved antiviral drugs are limited. Currently, FDA approved drugs primarily exist only for the treatment of influenza virus, HIV, hepatitis B virus, hepatitis C virus (HCV), and herpes simplex virus infections. The lack of available therapeutics for many pathogens, the potential for resistance to develop to existing interventions, as well as the difficulty in identifying effective antivirals have led to the need for the development of more robust, high-throughput screening assays (HTS) for the identification of novel antiviral candidates. For the purposes of this article, we are defining HTS as batch screening of libraries consisting of thousands of small-molecules or compounds against a specified target. These methods include selected or targeted screens [4], diversity and high-content screens [4,5], and the use of RNA interference (RNAi) [6]. Such assays have been used extensively for drug discovery in cancer research, which utilizes BSL-1/2 facilities. However, these assays present unique challenges when used in infectious disease research that require higher containment facilities and their implementation in these facilities has been limited.

There are over 50 different pathogens listed on the CDC Select Agent and Toxin registry, and the majority of these pathogens have no FDA-approved treatments or therapeutics [7]. Many of these pathogens are a major concern from a military and biodefense perspective, as these agents have the potential to be used as bioweapons and a number of these agents are endemic in areas where U.S. troops are deployed [8]. Therefore, development of effective therapeutics is a top priority for the military. Ribavirin has been suggested, and in some instances used, as a broad-spectrum antiviral drug for Crimean Congo hemorrhagic fever virus and Lassa virus (LASV) infections. However, there are toxicity issues with this compound and questions about its efficacy for these agents as well as a broad-spectrum treatment exist [9-11]. Therefore, the development of therapeutics for Category A agents is an important area of research and HTS methods are significant tools for this type of novel drug discovery. However, the nature of select agents dictates that most of these pathogens can only be studied in BSL-3/4 containment

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facilities. This presents potential problems for HTS assays used in these laboratories which include the limited ability to automate the screening process due to space constraints. In addition to the requirement for BSL-3/4 laboratory facilities, other challenges include regulatory issues (such as the rules and permits pertaining to work with select agents), the need for additional personal protective equipment (PPE), and the containment of equipment [12]. Therefore, the term HTS when used in the context of high containment laboratories is based on the ability to achieve higher drug screening throughput than with traditional assays with or without automation that would typically be observed in lower containment laboratories. In this article, we compare the various HTS methods and discuss the advantages and disadvantages of each in the context of BSL-3/4 laboratories. This article is not meant to be an exhaustive review of the literature; however, its purpose is rather meant to initiate a discussion about the most common HTS methods and the unique challenges they present to drug discovery for high-consequence pathogens as well as to highlight the potential for broader implementation of these procedures in BSL-3/4 facilities.

High-Throughput Screening Methods

Targeted/Selected screens

Targeted, or selected, screening is a method based on finding compounds, small molecules, or drugs that selectively bind to or inhibit a specific protein of interest. In infectious disease research, targeted screening focuses on finding a compound that will prevent specific binding or interactions with the pathogen protein of interest, whether they are host-pathogen or pathogen-pathogen interactions. This type of screening can be done multiple ways. One way is to do initial screens *in silico*, using three-dimensional (3-D) modeling, particularly if the crystal structure of the protein of interest is known [13,14]. Computer software can aid in the selection of compounds based on information from both the crystal structure of the target and libraries of both available and virtual compounds [15]. In addition to using the target in 3-D modeling, if there is a known ligand for the protein of interest, software can search the compound libraries for other compounds with similar properties and binding characteristics [13,14]. All of this information, in addition to looking at homology models and sequence similarities, can be used by the software to identify and select compounds with a high probability of interacting with the protein of interest [4]. This approach was used in the development of the anti-influenza drug Zanamivir [trade name: Relenza (GlaxoSmithKline)]. The crystallized structure of the hemagglutinin protein of influenza virus allowed computer software to model potential inhibitors of hemagglutinin, and resulted in the development of a safe and effective antiviral drug [16,17]. Another method for targeted screening is the screening of libraries of compounds against the protein of interest to find agonist compounds that promote protein-protein interactions or antagonist compounds that inhibit protein-protein interactions [18]. This type of screen can be done in multiple ways, including using cell-free systems, *in silico*, or pathway screening. An example of targeted screening is the small molecule library screen against heat-shock protein 70 (Hsp70). This technique identified novel, small molecule compounds that modulate Hsp70 activity during particular viral infections *in vitro* [19].

An advantage of targeted screening is that, depending on the technique used, the majority of the screening can be done in a BSL-1/2 laboratory or *in silico*. Because the infectious pathogen is not required, protein-protein interactions can be studied in a low-biocontainment laboratory. Some proteins of high-containment pathogens, such as

LASV and Ebola virus (EBOV), can be synthesized and used in a cell-free system, thereby maintaining BSL-1/2 compatibility. A second advantage of targeted screening is that the mechanism of action of the compound of interest is generally known, and if the molecular mechanism of action is unknown, then the protein target of the compound is known. This is valuable because the FDA requires a known mechanism of action for any potential new therapeutic drug [20]. In addition, this screening method minimizes the amount of time from conceptualization to testing in humans. The use of computer software can significantly increase the probability that identified compounds will be effective and extensive modifications for bioavailability may not be necessary prior to the first *in vitro* screens [3]. Finally, a major advantage of targeted screening is that it can make use of advances in sequencing of microbial genomes. The complete sequencing of a number of microbial genomes has identified conserved "target proteins" common across many pathogen families, genera, etc [21]. Targeting these conserved proteins could lead to the development of "broad-spectrum" therapeutics that would be effective against many different types of pathogens. For example, a small-molecule HTS identified a compound that was found to inhibit entry of several enveloped viruses in vitro, including filoviruses, arenaviruses, poxviruses, and HIV [22].

While there are many advantages to using targeted screening, there are also some drawbacks. One significant disadvantage is that only one protein is being screened at a time, lowering the number of total potential hits as compared to alternative screening methods. In addition, some viral proteins have been difficult to synthesize and produce in large quantities or require proteolytic processing for maturation. Another issue is that most viruses only encode about 8 to 15 proteins. This leaves these screens with a limited number of targets and the possibility that no hits will be found during a particular screen against the limited available targets. Additionally, while the identified compound may work in cell lines or in vitro, the compound may not be as effective *in vivo*. Finally, targeted screening relies heavily on knowledge about the protein of interest, including information about its structure, function, or binding ability. In some cases, this information is not known, or only partially known, which makes targeted screens more difficult to perform [4].

Diversity and high-content screens

Diversity screening is a method based on finding compounds, small molecules, or drugs that inhibit viral replication or pathogenesis at any level, and involves a much broader target base [4]. Instead of focusing the compound screen against one particular protein of interest, diversity screening identifies compounds that interfere with the pathogenesis of an agent at any point during infection. This is a simple methodology, with the basic concept of needing only the compound library, the pathogen of interest, cells to infect, and a suitable assay. Diversity screening has also driven the technology typically associated with HTS. Large libraries demand simple assays that can be run quickly as well as computers and robots that can handle the high throughput of samples and analysis of data. Available libraries used in diversity screening have included natural compounds [23,24], peptides [25], drugs [26], and synthetic compounds [3,24]. There is also the potential to screen solely with current FDA-approved drugs for potential drug repurposing [27]. Diversity screening has been used to identify candidate small-molecule inhibitors of yellow fever virus [28], dengue virus [26], and New World arenaviruses [29], though all are currently still in early testing and development.

High-content screening (HCS) is a diversity screening method that was developed when cellular imaging and analysis became automated.

This technique uses a fluorescence microscope that can image an entire well from a plate, allowing for visualization of multiple cells at the same time [30]. HCS has been utilized during all stages of the drug discovery process, from the primary small-compound HTS to deciphering the mechanism of action [30]. Instead of measuring one parameter like most HTS methods, HCS allows for measurement of multiple parameters, including shapes, textures, staining intensities, staining localizations, nuclear size, total number of cells, and percentage of virus-positive staining cells [5]. Most HTS assays cannot distinguish between infected and uninfected cells in the same wells, instead relying on the majority of cells in a well being infected and the assay to show significance between infected and uninfected wells. HCS can distinguish between these parameters and thus allows a more specific measurement of how a compound will affect viral replication. In addition, all these parameters, together with powerful computer algorithms, allow HCS to not only identify potential small-compound hits, but also aid in identification of toxicity and mechanism of action [5,30].

A major advantage of diversity screening is the possibility of discovering novel classes of drugs. This not only would increase the number of total available therapeutics for treatment but would also increase the number of different classes of therapeutics. This in turn could be pivotal in the challenges against the emergence of drug resistance. Also, because diversity screening does not focus on a single target, there are more potential targets available to achieve hits. Another advantage is that this type of screening identifies compounds that have a higher likelihood of efficacy *in vivo*. While both targeted and diversity screening methods identify potential compounds, hits identified by diversity screening are already known to inhibit pathogen replication and/or pathogenesis since an infectious pathogen is used in the screen. In addition, diversity screening is able to identify pathway inhibitors that a targeted screening technique might miss. Diversity screening also provides data sets with similar compounds, and the backbones of these compounds can be used by medicinal chemists to overcome potential problems associated with the result of the original structure, including poor solubility and increased toxicity.

While there are numerous advantages to diversity screening, this method does not often inform as to the mechanism(s) of action of identified compounds. Elucidation of mechanism of action is often quite difficult and requires substantial monetary and personnel investment. In addition, it is expected that the majority of compounds identified through screens will require multiple rounds of refinement before they are ready for clinical trials. This process can take years and the synthesis of thousands of derivatives to identify a molecule that is efficacious *in vitro* as well as in relevant animal models, and that is also safe for testing in humans [3]. Many pharmaceutical companies are unwilling to make the financial commitments that are necessary for this process for pathogens that may affect only a small number of individuals or for which there is no obvious commercial market. Also, while large libraries offer an enormous number of compounds to screen, they also result in huge data sets that are computationally challenging. In addition, some form of robotic automation is often needed to complete screens in a timely manner. A final hindrance to this screening method is the "vastness of chemical space." Chemists estimate that there are approximately 10^{40} possible drug molecules; however, most compound libraries do not exceed 10^6 molecules [4]. The goal of HTS is to identify novel candidates and the current numbers of most existing libraries are small in comparison to the possibilities, which limit the chances of finding novel classes of therapeutics to only those that are currently included in these libraries.

RNAi screens

RNAi was first described in *C. elegans*, and subsequently shown to be able to silence genes in mammalian cells in 2001 [31]. RNAi is accomplished by either using small-interfering RNA (siRNA) or short-hairpin RNA (shRNA) duplexes. The siRNA duplexes transfected into cells are very short, usually containing only 21-23 nucleotides, with one strand being complementary to the targeted cellular mRNA sequence. This siRNA incorporates into the RNA-induced silencing complex, binds to the target mRNA to induce endonucleolytic cleavage and degradation of the mRNA, and prevents the mRNA from being translated into protein [6]. Knockdown of the targeted cellular protein with siRNA is transient and depends upon the rate of cellular division, the target protein's half-life, and the siRNA-transfection efficiency [32]. Since their discovery, siRNA libraries that can target all known and predicted mRNA transcripts in humans have become commercially available [6]. Protein silencing can also be accomplished using shRNA. A DNA vector containing the shRNA sequence is transfected or transduced into the cell and a single RNA molecule is transcribed from this template. This RNA sequence has complementary regions separated by a short "loop" sequence which allows the RNA to fold back upon itself and form a "short hairpin" loop. This double-stranded RNA is then processed by cellular machinery, converting the shRNA into siRNA [33]. Recently, lentiviral vectors, based on the HIV genome, have been used to deliver shRNA into cell lines. These vectors are very effective because they can infect both non-dividing and dividing cells [32]. Genome-wide RNAi screens have been used to study the pathogenesis of HIV [34,35], HCV [36,37], West Nile virus [38], influenza virus [39,40], and *Mycobacterium tuberculosis* [41,42]. RNAi has also been proposed as a potential therapeutic when packaged in an appropriate vector [43,44]. For example, it was shown that lipid encapsulated siRNAs targeting various EBOV proteins given postexposure were protective against EBOV infection in non-human primates [45].

An advantage of genome-wide RNAi screens is that they can identify specific host proteins and pathways that pathogens require for successful replication. Current HIV entry inhibitors targeting CCR5, such as Maraviroc [trade name Selzentry (Pfizer)], demonstrate that host proteins can be viable drug targets [46,47]. In addition, RNAi-based therapeutics against HCV, HIV, and respiratory syncytial virus are currently in clinical trials [43,48]. The results of an RNAi screen can also lead to rational drug design. Because host factors required for replication are being identified, any currently available drugs that target those factors could potentially be used therapeutically. A second advantage is that the mechanism of action may be more rapidly uncovered than in a diversity screen, though not as rapidly as a targeted screen. Finally, comparison of different RNAi screens against the same pathogens revealed a very low overlap of commonly identified cellular factors. However, many of the same pathways were being identified, leading to the theory that targeting cellular pathways or networks may be more important than specific, individual proteins in many cases [49].

As with other screening techniques, there are also disadvantages of utilizing RNAi screens. First is the potential for identification of specific cellular proteins or pathways that are essential for host cell survival and function. These hits would not be helpful since targeting these proteins therapeutically could be more harmful to the cell than the infection and may not be relevant to the virus life cycle. A second disadvantage is that while host-pathogen interactions are being identified, no actual small-molecule inhibitor or compound is being identified. This means that once a cellular factor or pathway is identified, inhibitor compounds

may need to be developed *de novo*, unless they already exist or unless the candidate therapeutic is based on the RNAi. While identification of target proteins is a significant step forward, development of *de novo* compounds may require additional time, effort, and money, as compared to focused and diversity screens where a starting small-molecule is identified. Because these small RNAs can bind to the target mRNA, there is the possibility of non-specific binding to other mRNA molecules. This can lead to unintended down regulation of other proteins in addition to the protein of interest [50]. This can interfere with assay results because it can lead to false positives.

HTS in the Context of BSL-3 and BSL-4 Laboratories

In the context of high-consequence (BSL-3 and BSL-4) pathogens, there are many challenges that can arise during HTS. The requirements of BSL-3/4 facilities pose many unique challenges for the development and execution of the HTS assays. Anything that is used in a BSL-3 or BSL-4 laboratory must be inactivated before being removed from containment [51]. Inactivation usually relies on destruction or fixation of the pathogen or cells. This can create problems with HTS assay reading. For example, some assays depend on healthy cells to monitor cell toxicity and/or virus propagation. Inactivation would interfere with these assays. Some instruments are available in containment settings to provide better readouts of assays; however, space limitations are problematic in most facilities. Additionally, any equipment in containment must remain in containment unless properly decontaminated, which is a process that may damage or reduce the working lifespan of computers and other delicate electronics or optical systems. Typically, most institutions have utilized paraformaldehyde/bicarbonate decontaminations for equipment; however, this type of decontamination can be very damaging to most equipment. Switching to vaporized hydrogen peroxide, chlorine dioxide, and other methods will likely decrease damage to equipment and reduce the environmental impact of decontaminations [52,53]. The maintenance, repair, and updating of equipment in containment are also challenges that must be considered for HTS assays performed in containment. For example, the lack of fine-tuning dexterity in a BSL-4 suit may preclude detailed adjustments to equipment. However, new technologies continue to be developed to overcome these challenges.

A possible solution is to develop an assay that is not affected by pathogen inactivation methods so that the screens can be processed and read outside of containment. An example of this is the HTS assay developed for Nipah and Hendra viruses, two BSL-4 pathogens. This protocol was specifically developed to include cell fixation and virus inactivation in methanol to allow for the removal of plates from BSL-4 to perform immunodetection in BSL-2 [54]. In many cases, it is also possible to use formalin to fix plates of infected cells to perform additional screening steps or reads outside of containment [55]. Regardless of the type of inactivation performed, the assay must be validated to ensure that the inactivation does not interfere with any readings or results. While inactivation of samples allows for further evaluation of test results outside of containment, the additional burden to ensure inactivation and/or validate appropriate inactivation methods continue to be cumbersome.

Alternatively for some BSL-3/4 pathogens, developing assays which do not use wild-type virus or use a surrogate for first-level screening may be a viable option to keep the majority of work in a BSL-2 laboratory. Vaccine strains are often developed by serial passage of the virus multiple times in cell culture to attenuate the virus, and attenuated virus can be used at a lower biosafety level once the sufficient

safety testing has been performed. One example is the TC-83 vaccine strain of Venezuelan equine encephalitis virus (VEEV) that has been commonly used under BSL-2 conditions [56,57]. However, it is often unclear how the attenuation of the agent may affect the results obtained in screening assays. Put simply, it is possible that a compound found to be effective against the vaccine strain may not be effective against the wild-type pathogen, or that candidate compounds may be missed due to deletions or changes in the viral genome that occurred during the attenuation process. For example, TC-83 was attenuated by serially passing the virus 83 times in cell culture, resulting in mutations that significantly affect virulence [57], and in fact, differences in sensitivity to compounds have been noted in drug screens using virulent wild-type and VEEV vaccine strain (CES, unpublished data).

Pseudotyped pathogens, replication-deficient pseudotyped pathogens with reporter genes, or recombinant strains of pathogens can also be used for BSL-2 HTS. For example, this may involve developing a virus that has the envelope glycoproteins (GP) of the pathogen of interest but the genomic backbone of a lower containment pathogen, such as HIV or VSV [58]. An example of this is the development of pseudotyped viruses for LASV [18,59] and EBOV and MARV [55,58]. The pseudotyped viruses, HIV/EBOV-GP (Ebola GP on a HIV genomic backbone) and HIV/LASV-GP, can be studied in BSL-2 containment [55,59]. However, a potential major disadvantage of the pseudotyped pathogen is that they may have a different morphology than the parental strain. For example, EBOV is filamentous while the pseudotyped HIV/EBOV-GP is spherical [55]. This morphological difference may lead to false positives and false negatives during HTS because of differences in binding properties and potentially entry pathways [60]. In addition, these pseudotyped pathogens only allow for screening of candidates that target the GP(s) and are as such primarily of use for screens designed to look at viral attachment or entry.

Complementary to pseudotyped pathogens, minigenome systems may be used to study replication and transcription of high-consequence pathogens at a lower biocontainment level. Minigenome systems utilize a viral genome where all or some of the viral open reading frames have been removed and replaced with a reporter gene [61]. In viruses with segmented genomes, a minigenome system is usually based solely on a single genome segment [62,63]. The non-coding regions of viral RNA responsible for replication and transcription are retained so that these regions can be the templates for the viral polymerase. These minigenome viral RNAs can either be transfected directly into host cells or utilized with a T7- or RNA polymerase I-driven system to generate the minigenome RNA in the host cells [61,62]. The minigenome system allows for the study of viral replication, transcription, and in some cases, packaging, through the amplification of the reporter gene [61]. These systems have been utilized to study EBOV [64,65], Nipah virus [66], arenaviruses [67,68], and bunyaviruses [63,69,70]. Unfortunately, as is the case with pseudotyped viruses and vaccine strains, there is no evidence that all potential hits from HTS will work efficiently on the wild-type virus. Since minigenome systems are missing a complete genome and associated viral components, this may lead to identification of false positive or false negatives during HTS screening.

HCS has recently been used to screen libraries ranging from 500 – 5000 small-molecules for inhibitors of EBOV [5], MARV [5], HCV [71], and dengue virus [26]. These assays have the ability to combine cytotoxicity and efficacy assays into a single assay. Additionally, in one assay, HCS can specifically determine the percentage of infected cells and has the potential to define a mechanism of action. Monitoring multiple parameters in a single assay allows for a minimization of

Method	Advantages	Disadvantages	BSL-3/4 Limitations
Targeted Screen	• Can be done in BSL-1 • Mechanism of action is known • Can find "broad-spectrum" targets	• Lower number of hits • Must have more information about protein of interest • Limited number of targets	• Does not have to be done in BSL-3/4, unless wild-type virus is used
Diversity Screen	• More available targets • Can find "novel" targets • Can use infectious virus	• No mechanism of action • Longer time until potential drug is available • Libraries can be expensive	• Equipment (robotics, assay readers, etc.) must be in BSL-3/4 • Appropriate PPE required • Decontamination of samples is required before removal from lab
RNAi	• Can target specific host proteins and pathways • Mechanism of action may be known • Can use infectious virus	• Pathways identified can be essential to host cell survival • Drugs developed after identification of pathway, costing more time and effort	• Screening equipment (robotics, assay readers, etc.) must be in BSL-3/4 suites • Appropriate PPE required • Decontamination of samples is required before removal from lab

Table 1: Comparison of high-throughput screening methods.

experimental steps and is a huge advantage when working in the highly constrained environment of BSL-3/4 [5,30].

All of these methods have advantages and disadvantages that should be taken into consideration (Table 1). While there is no screening method currently available that eliminates the need for work with high-consequence pathogens in BSL-3/4 laboratories, targeted screening has the potential to minimize the amount of work that is done in high-containment laboratories. Individual proteins can be synthesized and used in BSL-1/2 settings, allowing work to be performed for less monetary investments and at a rate that exceeds what is often achievable in BSL-3/4. In addition, by creating first level screens outside of containment, the risk to personnel is reduced. The majority of the screening can be done in this context and subsequent hits can then be tested against live pathogens in cell systems followed by *in vivo* studies as warranted. In contrast, diversity and RNAi screening have the ability to significantly increase the amount of novel therapeutics and targets against pathogens. It is important to note that the aforementioned methods are extremely useful and cost effective to down-select large libraries to "hits" that will be further tested with authentic virus assays.

Conclusions

In summary, there are multiple ways to utilize HTS to identify potential therapeutics for high-consequence pathogens. The requirement to study these pathogens in BSL-3/4 containment facilities creates many challenges for researchers working in this environment. While the limited resources of containment facilities has been a major impediment for drug discovery and development against potential biothreat agents, recently there has been an increase in the number of available BSL-3/4 laboratories [72]. In addition, new facilities with better laboratory design, such as open and flexible floor plans, and novel decontamination methods, will facilitate more HTS screening by making space available for robotics. To date, standard Class II and Class III biological safety cabinets have been utilized in BSL-3/4 laboratories. Robotic cabinet designs have been precluded due to space constraints; however, in newer facilities, the space is significantly increased and the utilization of robotics will become possible. Also, with advances in technology, refinement of robotics, miniaturization of equipment, and reductions in cost, the use of effective HTS methods in BSL-3/4 is slowly increasing.

In the last decade, a variety of screening methods have identified promising compounds that could potentially be developed into antivirals for the treatment of some high-consequence pathogens.

For example, the compound ST-193 was discovered during a targeted screen against LASV-GP using a pseudotyped LASV [59]. It was shown to inhibit arenaviral entry and subsequently demonstrated to increase the survival rate of LASV-infected guinea pigs [73]. Additionally, the compound ST-246 was discovered during a diversity screen against Vaccinia virus [74]. It was subsequently shown to prevent egress of monkeypox virus and has conferred protection against the disease in nonhuman primates [75]. Small-molecule screens demonstrated that the cellular protein Niemann–Pick C1 was required for EBOV infection. Small-molecule inhibitors of Niemann-Pick C1 were then shown to inhibit EBOV infection *in vitro* [76,77]. As discussed previously, lipid encapsulated siRNAs targeting various EBOV proteins were protective against EBOV infection in nonhuman primates when given immediately postexposure [45]. Finally, phosphorodiamidate morpholino oligomers (DNA analogs that target mRNAs and block translation) that target the filovirus protein VP35 were shown to efficiently protect mice against lethal EBOV infection [78].

While progress is being made, these and other promising compounds are still in the early phases of testing and development and are not yet ready for FDA-approval. Therefore, a single method approach may not be enough to significantly advance drug discovery, so parallel screening approaches that combine different methodologies simultaneously are necessary. Drug discovery using HTS is a powerful tool and continual improvements in assay development and available BSL-3/4 space remains important for the development of new therapeutics against many high-consequence pathogens.

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