Lentiviral high-throughput assay shows promise for identifying novel HIV-1 inhibitors

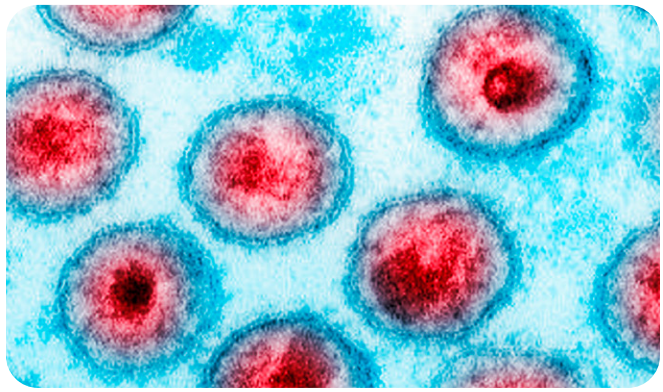
The human immunodeficiency virus (HIV) attacks a person's immune system, specifically CD4+ T cells. The virus destroys these cells, subsequently weakening the infected individual's immunity against opportunistic infections. If HIV is not treated, it can lead to the development of acquired immunodeficiency syndrome (AIDS).

The HIV epidemic remains a major global health challenge – in 2020 alone, 37.7 million people were estimated to be living with the virus and around 680,000 people died from HIV-related causes.[1] Despite significant progress in the field, there is currently no cure or effective HIV vaccine available.

HIV is a retrovirus, which means it carries single-stranded RNA as its genetic material and requires reverse transcription for the integration of its viral genome into the host genome. HIV uses a complex series of steps to deliver its genetic material into the host cell, while simultaneously evading the host immune response. First, the HIV envelope protein binds to the primary cellular receptor CD4 and then to a cellular coreceptor. This sequential binding triggers fusion of the viral and host cell membranes, initiating release of the HIV capsid into the cell. Once the viral capsid has entered the cell, reverse transcriptase converts the viral RNA into pro-viral DNA, which can then be integrated into the host cell genome by the integrase.

Antiretroviral treatment

While there is currently no cure for HIV, medicines have been developed to prevent the virus from replicating and to slow progression and transmission of the disease. At the forefront of these medicines are reverse transcriptase inhibitors, of which there are two subclasses. The first are nucleoside analog reverse- transcriptase inhibitors (NRTIs),



which were the first class of antiretroviral drugs for HIV to be approved by the FDA, and the second are the non-nucleoside reverse transcriptase inhibitors (NNRTIs). An antiretroviral (ARV) regimen for a treatment- naive patient generally consists of two NRTIs administered in combination with a third ARV drug from one of three drug classes: an integrase strand transfer inhibitor (INSTI), a NNRTI, or a protease inhibitor (PI).[2]

While antiretroviral therapy (ART) is one of the most effective tools in the current fight against HIV, the emergence of drug resistance is proving to be a significant challenge in treating the condition. HIV reverse transcriptase lacks the proofreading capabilities inherent in many cellular polymerases, meaning that its duplication of the HIV genome is highly error prone. Indeed, it has been suggested that HIV-1 has a 100,000-fold higher mutation rate per base and replication cycle than yeast.[3] This means that HIV frequently generates escape mutations, which constitutes a major obstacle toward effective treatment.

The World Health Organization (WHO) reports that up to 26% of people initiating ART are infected with a virus carrying resistance to first-line drugs, and very high levels of drug resistance – up to 69% – are seen in infants born to mothers

revvity

infected with HIV.[4] Many in the field believe that HIV drug resistance could jeopardize the efficacy of ARTs, resulting in increased numbers of HIV infections and HIV-associated morbidity and mortality worldwide.

Identifying novel HIV-1 inhibitors

Due to the challenges of HIV therapy mentioned above, researchers are looking for novel and innovative approaches to develop HIV therapies. One promising avenue is the development of new small-molecule drugs against targets associated with a low likelihood of resistance development. However, the limited number of proteins encoded by HIV, coupled to their extensive application in previous screening programs and high mutation rate, make them less attractive for novel drug discovery approaches.[3] An alternative approach is to shift the focus away from inhibiting viral proteins, and instead develop modulators against the host proteins that facilitate HIV pathogenesis and disease. A large number of viral and cellular protein–protein interactions are necessary for viral infection, and it would be expected that these cellular factors, which are essential for HIV infection or replication, would have a much lower intrinsic mutation rate than the virus itself.

With this in mind, Bernhard Ellinger, from the Department ScreeningPort, Fraunhofer Institute for Molecular Biology and Applied Ecology IME in Germany, and Kristoffer Riecken, from the University Medical Center Hamburg-Eppendorf (UKE) in Germany, along with a team of researchers, set out to identify novel inhibitors of HIV-1. Specifically, they developed a high- throughput HIV-1 screening platform based on lentiviral vectors that targeted both viral proteins and all cellular partners involved in HIV-1 un-coating, intracellular trafficking, reverse transcription, nuclear entry, and genome integration.[3] When asked how their collaboration transpired, Riecken explained that he had already been working on lentiviral vectors for gene transfer, which opened the door to the partnership with the ScreeningPort. “Since the vectors were derived from HIV-1, they mimicked quite a large part of the viral life cycle,” he said.

Assay development and HTS

One of the first challenges that the team faced was to establish a high-throughput screening (HTS) assay that was biosafety level (BSL)-1 compatible. Biosafety levels dictate the type of work that is allowed to take place in a lab setting and they are designed to protect laboratory personnel, as well as the surrounding environment and community. These levels, which are ranked from one to four, are selected based on the agents or organisms that are being researched or worked on in any given laboratory setting. “Our lentiviral vectors are always used under BSL-2; however, the Fraunhofer screening facility was BSL-1 so we had to step down a level,” said Riecken.

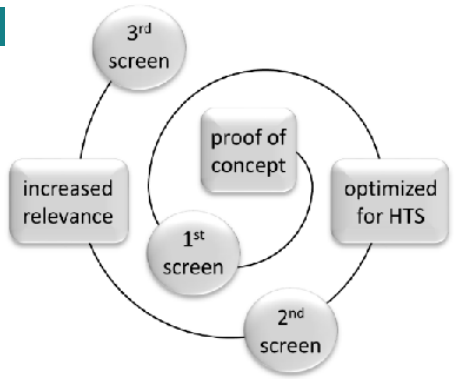
The team achieved this by pseudo-typing HIV-1-derived lentiviral vectors with the envelope protein of ecotropic mouse leukemia virus (MLV), which is unable to bind to human cells. These “mouse only” viral particles could still be used in human cells after the murine Cat1 symporter, which serves as the receptor for ecotropic MLV, was stably expressed in HEK293T cells. “This was quite a nice trick because as soon as uptake of the particles was successful, the further reverse transcription, intracellular trafficking, and integration was based on the HIV molecules,” said Riecken.

To develop a physiologically relevant assay that was compatible with HTS, the researchers underwent three fundamental stages of assay development and stepwise improvement (Figure 1). First, they conducted a fluorescence-based proof-of-concept screen where cells were co-transduced with two types of lentiviral particles (one depending on the wild-type HIV-1 reverse transcriptase and the other on an AZT-resistant reverse transcriptase mutant) and imaged using a high-content screening system. Riecken noted that this screen revealed to them that fluorescent proteins were not the most efficient way to do HTS. “The readout was too slow and it’s also expensive,” he said.

To improve the throughput and stability of the assay, while simultaneously reducing costs per well, the team decided to replace the fluorescence readout with a luciferase-based system for their second screen. Cells were seeded in 384-well plates and luminescence intensity was measured using an Envision multimode reader. “The readout was much faster,” confirmed Ellinger. “Switching to luciferase meant we could analyze full 384- well plates within a few seconds.”

Finally, the team set out to increase the biological/ pharmacological significance of their screening platform by adapting it to human T cells, which are the natural target of HIV-1. For this, they generated a PM1 cell line which stably expressed the mCat1 receptor. The researchers explained that the PM1-mCat1 cell line is a derivative of the human T cell line PM1, which is a standard cell line broadly used in research of HIV biology. “This enabled us to go on and perform a large-scale screening using industrial standards and a relevant cellular model, which would allow us to deliver meaningful data,” said Ellinger.

A



B

	Cell line	Read out
1st Screen	Human HEK293T-mCAT1	Fluorescence
2nd Screen	Human HEK293T-mCAT1	Luminescence
3rd Screen	Human T cell PM1-mCAT1	Luminescence

Figure 1: Overview of the assay methodology development. (A) Representation of the assay development based on incremental improvement. (B) Comparison of the screening methodology in terms of relevance, based on a traffic light system. Figure taken from Ellinger et al.[3]

Having developed a physiological relevant assay compatible with HTS, the researchers divided a 200,640-compound in-house library into 3,000 clusters based on chemical diversity. Compound plates were chosen according to the maximum number of clusters per plate, resulting in a screening collection of 26,048 compounds from 2,723 clusters. "This similarity clustering drastically reduces the number of compounds you have to test without compromising too much chemical diversity," explained Ellinger. "It is quite an effective approach to get additional structure-activity relationship data and learn something new about the compounds, potentially finding even more active ones." The researchers report that the screen yielded z' values greater than 0.8 with a hit rate of 3.3% and a confirmation rate of 50%.

Next, they selected 93 hits and enriched the collection with 279 similar compounds from the similarity clustering performed previously to identify promising structural features. Finally, the most active compounds were validated using orthogonal assay formats.

Ellinger explained that one of the key advantages of their platform, in addition to being BSL-1 compatible, is that it is an unbiased method to screen for novel HIV-1 inhibitors. "We can even screen for drugs where the target has not been known to be relevant for HIV biology so far," he said. "There's also some quite technical aspects to [our platform] and we used a number of different technologies. We started with microscopy in 96-well plates and then we went for miniaturization and automatization. We also used multimode plate readers and automated dispensing for liquids and compounds."

Although their screen was a success, their study was not without its challenges. "As always, money was the biggest problem with these kinds of projects – whatever you're doing it's going to be expensive," said Riecken. "We applied for funding several times – it took years to get funding to do some of the larger experiments." Ellinger added that translating protocols from one site to another also proved challenging. "One shouldn't underestimate the complexity of translating findings from one lab to another. We found we'd have a perfectly running protocol at Kristoffer's site, but it wasn't working at ours, or the results weren't the same, or we had unexplained shifts in our data."

Next steps

Looking forward, the researchers are hoping to identify molecules that are specific for difficult to treat strains of HIV. "We've cloned these multiple resistant strains, for example where the reverse transcriptase or integrase has several mutations, which can then be used as the vector. These can then be used for screening to identify molecules that are specific to these mutant strains," said Riecken. He added that at the University Medical Center Hamburg-Eppendorf, researchers are also looking at gene therapy against HIV, potentially curing the disease. However, he noted that gene therapy against HIV is still experimental and quite an expensive treatment. "As long as there are effective drugs available, they're usually easier to use, cheaper, and also suitable for a much larger patient cohort, also in low- and middle-income countries."

Ellinger concluded that it's also important to consider the patient population when developing drugs against HIV. "You have young people, elderly, but also children and infants. Currently, children are treated with slightly different combinations than adults, but this is not really ideal. This is something which can be improved. You ideally want to have a drug or a combination which somehow works well on all patient groups, especially in healthcare systems with lower resources."

Bernhard Ellinger

Bernhard studied Biochemistry at the Martin Luther University Halle-Wittenberg, Germany before joining the group of Prof H. Waldmann at the department of Chemical Biology at the Max-Planck Institute in Dortmund, Germany, for his PhD. After finishing his PhD on modulators of the wnt signaling pathway he moved to Hamburg for a position at the European ScreeningPort GmbH. Here he worked on numerous projects in the area of assay and drug development. Today, Bernhard is a Senior Scientist at Fraunhofer IME ScreeningPort where he is responsible for advanced cellular and biochemical screenings. The assays he is performing are dealing with natural products and synthetic libraries, but also include enzymatic characterizations. They are covering areas such as enzyme activity, toxicology, cellular signaling, target deconvolution, and antiviral and antibacterial screening. Bernhard has published 38 articles about various topics in Chemical Biology and has an h-index of 18.

Kristoffer Riecken

Kristoffer studied Biochemistry and Molecular Biology at the University of Hamburg, Germany with his Diploma thesis carried out at the Heinrich-Pette- Institute for Experimental Virology in Hamburg, Germany, where he worked on lentiviral vectors for the first time. For his PhD thesis he joined the Group of Boris Fehse at the University Medical Centre Hamburg- Eppendorf, Germany. After about two years at the Cincinnati Children's Hospital Medical Center, USA and the University of Frankfurt, Germany, he came back to the Research Department Cell and Gene Therapy of Boris Fehse. As an expert in lentiviruses and lentiviral-vector technology, he has invented the LeGO vectors for ectopic expression or down-regulation of multiple genes, RGB Marking for multi-color clonal cell tracking in microscopy and Optical Barcoding to track and quantify fluorescently labeled cell clones by flow cytometry. He now works on chimeric antigen receptors and appropriate viral vectors for their use in gene therapy. His expertise in viral vectors lead to the development of screening assays for HIV- and SARS-CoV-2 inhibitors.

References

1. HIV/AIDS [Internet]. Who.int. 2021 [cited 20 October 2021]. Available from: https://www.who.int/news-room/fact-sheets/detail/hiv-aids

2. What to Start: Initial Combination Regimens for the Antiretroviral-Naive Patient | NIH [Internet]. Clinicalinfo.hiv.gov. 2021 [cited 10 November 2021]. Available from: https://clinicalinfo.hiv.gov/en/guidelines/adult-and-adolescent-arv/ what-start-initial-combination-regimens-antiretroviral-naive

3. Ellinger B, Pohlmann D, Woens J, Jäkel F, Reinshagen J, Stocking C et al. A High-Throughput HIV-1 Drug Screening Platform, Based on Lentiviral Vectors and Compatible with Biosafety Level-1. Viruses. 2020;12(5):580.

4. Fact Sheet: HIV Drug Resistance [Internet]. Who.int. 2021 [cited 20 October 2021]. Available from: https://www.who.int/newsroom/fact-sheets/detail/hiv-drug-resistance.