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[1] **Type**: Use one of the following codes (in consistence with the Description of the Action):
R: Document, report (excluding the periodic and final reports)
DEM: Demonstrator, pilot, prototype, plan designs
DEC: Websites, patents filing, press & media actions, videos, etc.

[2] **Dissemination level**: Use one of the following codes (in consistence with the Description of the Action)
PU: Public, fully open, e.g. web
SEN: Sensitive, limited under conditions of the Grant Agreement

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Summary

1. Introduction

Work package 6 comprises the identification of small molecules that inhibit cellular uptake of Marburg Virus (MARV) and of antigen candidates for the development of improved MARV vaccines. In order to establish a working pipeline against MARV and future viral threats, a key step is the opportunity to screen large amounts of possible drug candidates in a reasonable short amount of time. Therefore, a robust automated screening protocol needs to be established.
Since MARV and host cells interact via different viral ligand - host receptor pairs, different setups with minor adaptations to the original screening assay will be needed, in order to address the diverse targeted interactions. In more detail, we will search for phosphatidylserine (PS) to PS-receptor inhibitors, first in Huh-7 cells expressing TIM-1. Further we will investigate the binding of glycoproteins (GP) and lectins (e.g. DC-SIGN). The last approach comprises new possible MARV GP target sides which will be identified during the project and we will search for inhibitors of such protein – protein interactions.
The aim of the first year within WP6 was the establishment of a screening protocol, that is feasible, practicable and versatile for the identification of MARV entry inhibitors. The results obtained from the different screening campaigns will be used by other members of the consortium to establish better models and to develop new structure-optimized inhibitors. All compounds which show an inhibitory effect within the initial BSL-2 surrogate system will be evaluated with authentic MARV under BSL-4 conditions.

2. Description of Activities

2.1 Screening assay development

An automated screening assay was established with a focus on providing throughput and stability during planned screening campaigns (Overview is given in **Figure 1**). To be able to handle large libraries of compounds (>100.000) in reasonable amounts of time and with limited compound consumption, the final assay volume should not exceed 50 µl and make use of the 384-well-plate format. Therefore, we determined the optimal number of cells to be seeded in a single well within a 384 well plate as 5.000 Huh-7.5 (obtained from WP1) viable cells in DMEM containing 3% FBS.

At the beginning, two BSL-2 surrogate systems (rVSV-MARV-GP encoding for GFP virus and a rVSV-MARV-GP-miniGFP-HiBiT luciferase virus) were tested for the signal strength of the GFP infection reporter. Cells infected with the split luciferase-encoding virus did not show detectable fluorescent signals on our microplate reader, and the addition of substrate to detect luciferase activity would generate extra costs. We therefore decided to work with the rVSV-MARV-GP GFP virus in the initial stage, which showed detectable GFP signals.

According to the fluorescent readout signal, plates with black borders (avoiding light scattering to adjacent wells) and transparent bottom (for readout through the bottom layer due to the adherent cell type) have to be used. The recombinant rVSV-MARV-GP pseudotyped viruses (obtained from WP1) showed high infection rates reaching titers >1x10^7. Therefore, a dilution factor of 1:10.000 was used for infecting cells in DMEM containing 3% FBS.

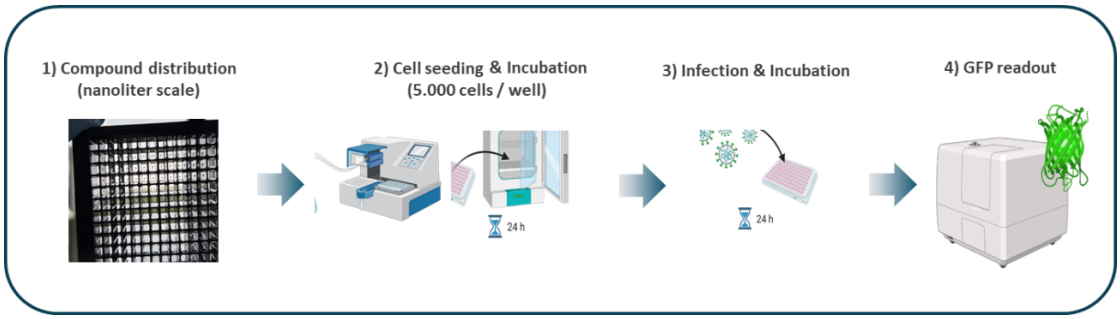


Figure 1: Screening method overview. Compounds to be investigated will be distributed via an acoustic dispenser in nanolitre scale (1). Next, cells will be seeded and incubated together with the compounds for 24 h (2). On the next day, compounds will be challenged for entry inhibition effect via virus infection (3). Entry inhibition will be determined due to absence of fluorescent signal (4) after 24h of infection. (Graphics created with BioRender.com)

2.1.1 Initial screening protocol

1. **Distribution of compounds**

- Compounds are transferred from source plates to screening plates (Nunc 142761) via an acoustic dispenser (Labcyte Echo 550). The preferred dilution of the EU-OPENSCREEN library during initial screens is 50 µM.
  The typical DMSO stock solution concentration is 10 mM. 250 nl of stock solution equals 50 µM at 50 µl final assay volume and 0.5% DMSO concentration.

2. **Seeding of cells**

- Cells were grown according to the SOP 'Combine_SOP_Huh7_cells' from February 2025 (Annex 1.) in T75 cell culture flasks until they reached 70 % confluency. For screening, cells were washed once with PBS and treated with 1 ml of trypsin/EDTA for 5 min at 37°C. Subsequently, cells were transferred to fresh DMEM 3% FBS containing media (1 ml cell suspension + 9 ml fresh media) and washed once with 10 ml new media.
- Count viable cells using a LUNA II cell counter (stain 1:1 with 0.4% Trypan Blue solution).
- For seeding: dilute 5.000 viable cells / well in 45 µl DMEM containing 3% FBS.
- Distribute cells using the MultiDrop dispensing unit within a Beckman Coulter i7 robot system surrounded with a sterile hood allowing BSL-2 work.
- Let cells seed for 24 h at 37°C in Cytomat 5 incubator with 90% humidity and 10% CO_2. Thus, cells will reach 90% - 100% confluency for infection on the next day.

3. **Infection of cells**

- Dilute rVSV-MARV-GP stock (prepared in WP2 according to protocol Annex 2.) aliquot 1:1.000 in DMEM 3% FBS containing media.
- Infect cells with 5 µl of diluted virus using CyBio Selma (96-Head) equipped with 25 µl filter tips.
- Incubate infected cells for 24 h at 37°C in Cytomat 5 with 90% humidity and 10% CO_2.

4. **Detection of fluorescence**

- Use i7 robot program: "Combine day 3"

- The program will transfer the plate from the Cytomat to Synergy H1 reader and back to the respective home position after reading.
- Measurement: 485 nm excitation and 512 nm emission wavelength at 1 mm height (plate bottom) with fixed gain setting to 99%.
- Results will be written in Excel-format and saved as (PlateBarcode_Timestamp_Date_Experiment).

5. **Hit picking and validation**

- A positive value for the entry inhibitor screen is considered a value of at least 50% inhibition of the fluorescent signal. A possible hit will be further validated via dose-response analysis, and cell viability will be checked using an ATP dependent cell-titer glow assay.

3. Results

3.1 Optimal signal-to-noise ratio & positive control

The GFP reporter readout was found to give an optimal signal-to-noise ratio of 1:2.5 at 24 hours post infection using 485 nm excitation and 512 nm emission wavelength (monochromator GFP setting) with a fixed gain of 99% on a Synergy H1 plate reader (**Figure 2**).

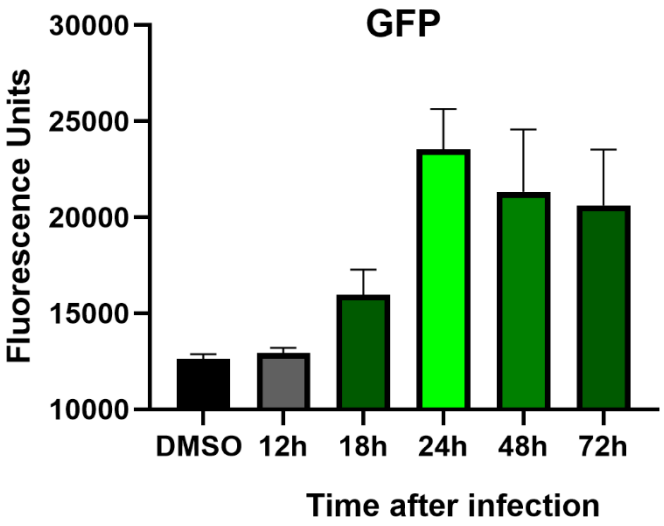


Figure 2: GFP-Signal readout obtained after 12 hours of infection until 72 hours after infection.

As a pharmacological positive control inhibitor, promethazine hydrochloride was chosen according to a screening assay conducted by Rong *et al.* in 2015, which showed inhibitory effect against MARV and Ebola virus. The Test-Layout is given in Figure 3. We observed maximum inhibitory effect at 5 µM and no cytopathic effects on host cells at this concentration (**Figure 4 A & B**). Specificity of MARV-GP inhibition by the control compound was shown using VSVΔG-GFP as control virus using the same test layout **(Figure 5 A & B**).

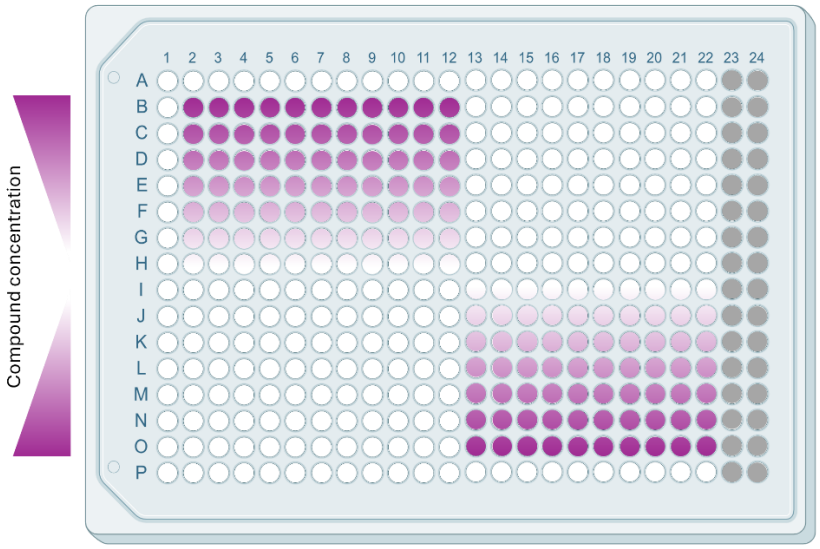


Figure 3: Test-Layout for positive control inhibitor tests. The MARV GP inhibitor promethazine hydrochloride (purple) was used in following concentrations: 20 µM (darkest color), 10 µM, 5 µM, 2.5 µM, 1 µM and 0.5 µM (lightest color). White cells contained 100 nl DMSO instead of compound. Cells A1 until P22 got infected with virus. Grey cells (A23 until P24) served as negative control, without viral infection.

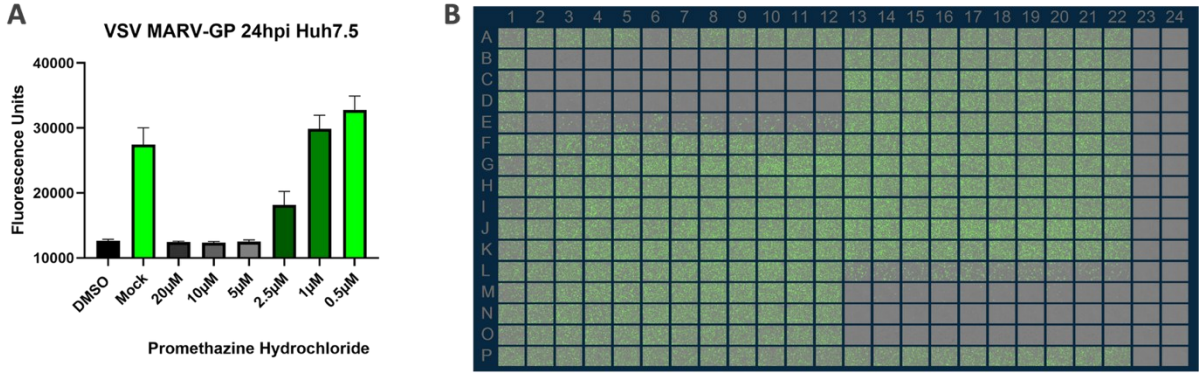


Figure 4: GFP-Signal readout obtained using promethazine hydrochloride as control compound against rVSV-MARV-GP infection. Relative fluorescence unit read-outs obtained from Synergy H1 plate reader 24 hours post infection (A). Plate overview (bright field and GFP overlay) obtained within an Incucyte instrument at 10X magnification (B).

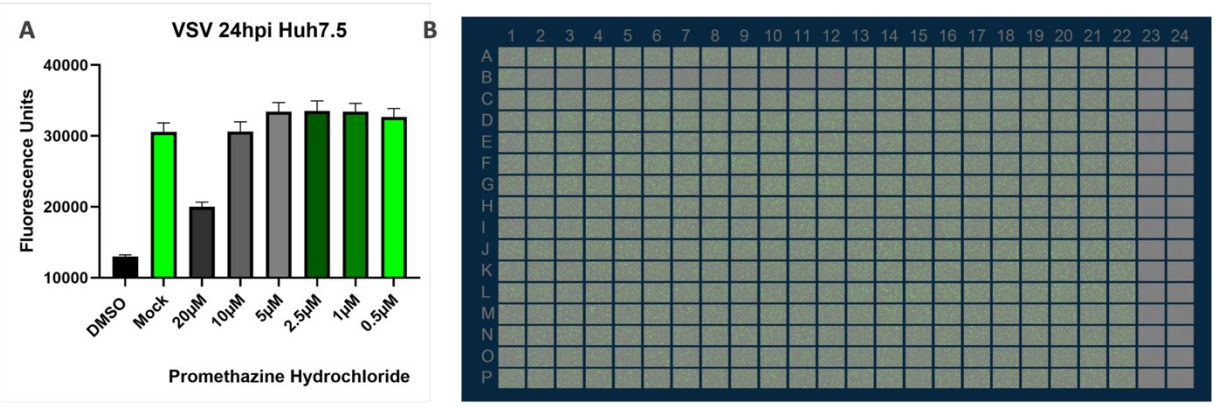


Figure 5: GFP-Signal readout obtained using promethazine hydrochloride as inhibition control compound infected with VSVΔG-GFP. Relative fluorescence unit read-outs obtained from Synergy H1 plate reader after 24 hours (A). Plate overview (bright field and GFP overlay) obtained with Incucyte at 10X magnification (B).

3.2 Z´-factor

The Z'-factor is a measure to assess the quality of a screening assay. For the above-described method it was found to be at least 0.67, rendering the assay as excellent according to Zhang *et al.* 1999, where a value between 0.5 and 1 is considered optimal (for individual values and standard deviations see figure 6). Using our settings, compounds can be tested according to standard protocol for primary screening (50 µM) and detection of positive readouts (hits) at 50% inhibition.

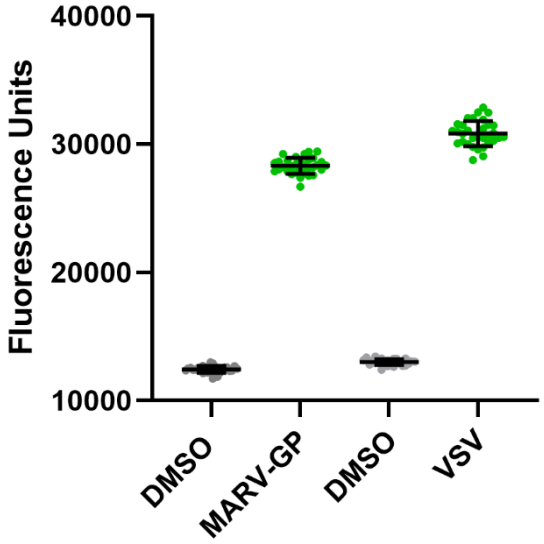


Figure 6: Plot of individual values from positive and negative controls shown in Figures 3B and 4B. Z'-factor for first plate DMSO (negative) and MARV-GP infected cells (positive) was calculated 0.83. For the second plate DMSO (negative) and VSV infected (positive) it was calculated 0.67.

3.3 Throughput

The automated system can run 10 - 20 plates/day depending on host cell growth and/or differentiation status ($2x10^6$ cells/plate needed). Cell seeding is done via a Multidrop dispensing unit in 45 µl of DMEM containing 3% FBS, 24 hours prior to infection under sterile conditions. Infections were carried out using a pipetting robot (Analytik Jena CyBio Selma) equipped with a 96 multichannel head under BSL-2 conditions.

4. Conclusion

We have tested two BSL-2 MARV GP pseudotyped VSV pseudovirus strains on Huh-7 cells and observed a strong GFP signal after 24 hours of infection with rVSV-MARV-GP. Then we established a positive control compound, which shows complete inhibition of viral infection at 5 µM. We also calculated a Z'-factor of at least 0.67, showing that the established method is capable for high throughput screening. With regard to high throughput, we have established a robust screening protocol during the first months of the project and achieved the goal D6.1.

5. References

Cheng H, Lear-Rooney CM, Johansen L, Varhegyi E, Chen ZW, Olinger GG, Rong L. 2015. Inhibition of Ebola and Marburg virus entry by G protein-coupled receptor antagonists. J Virol 89:9932–9938. doi:10.1128/JVI.01337-15.

Zhang JH, Chung TD, Oldenburg KR, A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 1999;4(2):67-73.

6. Annexes

STANDARD OPERATING PRORCEDURE

Cultivation of Huh-7 cells

Purpose and Scope

This standard protocol for the cultivation of Huh-7 cells is intended to ensure that all members of the COMBINE-MARV consortium treat the cells in the same way to avoid changes in the cells due to different handling. These instructions are only aimed at the cultivation of the cells; separate SOPs will follow for the individual experiments.

Huh-7 cell line

Origin

Huh-7 cells are established in 1982 from a well differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumor in a 57-year-old Japanese male [1].

Characteristics

Huh-7 cells display an epithelial-like morphology, with a hexagonal shape, and adhere to surfaces to form 2D monolayers during growth. The majority of Huh-7 cells show a chromosome number between 55 and 63 (mode 60) and are highly heterogeneous. Although containing many mutations and INDELS, it is worthy to note the Huh-7 cells have a point mutation in the p53 gene [2].

Materials

Material	Producer	Note
Cell culture flask, T-75, surface: Standard, filter cap	Sarstedt 83.3911.002	
Cell culture flask, T-25, surface: Standard, filter cap	Sarstedt 83.3910.002	
Dulbecco's Modified Eagle Medium (DMEM) high glucose, GlutaMAX supplement	Gibco™ 61965059	
Fetal Bovine Serum (FBS)	Sigma-Aldrich F7524	heat inactivation 30 min at 56°C
Trypsin-EDTA (0.05%), phenol red-20 x	Gibco™ 25300096	
PBS, pH 7.4	Gibco™ 10010015	
Dimethyl sulfoxide (DMSO) Cell culture grade	PanReac AppliChem A3672	

Procedure

Growth medium

DMEM (high glucose, GlutaMAX supplement) with 10% h.i. FBS.
Alternatively, you can also use DMEM without GlutaMAX, but then add 5mM L-glutamine.

Thawing

Thaw frozen cells rapidly in a 37°C water bath.

- Thaw cells in water bath, transfer into a 15 ml tube and dilute to 5ml total volume using growth medium
- Centrifuge at 1200 rpm (250xg), 5 min at room temperature
- Discard supernatant and resuspend the pellet in 5 ml growth medium
- Seed a 25 cm^2 flask with the entire volume of resuspended cells

Cultivation

Huh-7 cells are incubated at 37°C with 5% CO_2.

Renew medium 2-3 times a week, and maintain cell confluence between 30-90%. Do not allow cell concentration to exceed 90% confluence, otherwise cells will decrease proliferation rates.

- Discard the medium of a confluent T75 flask
- Carefully wash the cell layer with 10 ml PBS (do not apply PBS directly to the cells)
- Discard PBS
- Add 2 ml trypsin and incubate at 37°C for up to 10 minutes until the cells detach
- Fill up with 8 ml growth medium and gently resuspend the cells
- Collect the 10 ml cell suspension
- Count cells for experiments and seed at the required concentration
- For cultivation, 1 ml of the cell suspension is added to 9 ml of fresh growth medium in a new T75 flask (if the cells grow too quickly or too slowly, the cell suspension can be adjusted)

Seeding for Experiments

Huh-7 cells have a doubling time of 24 hours under good condition. For experiments, it has proven useful to use a lower FBS concentration of 3 %.

For transfection or infection seed $3{,}5x10^4$ cells in 24-Well, $2x10^5$ cells in 6-Well or $1x10^6$ cells in T75 Flask for a confluence of 60% at next day. A higher confluence leads to a lower transfection capacity.

Freezing

Huh-7 cells can be frozen in a growth medium supplemented with 10 % DMSO.

References

1. Nakabayashi, H., et al., *Growth of human hepatoma cells lines with differentiated functions in chemically defined medium.* Cancer Res, 1982. **42**(9): p. 3858-63.
2. Kawamoto, M., et al., *Identification of Characteristic Genomic Markers in Human Hepatoma HuH-7 and Huh7.5.1-8 Cell Lines.* Front Genet, 2020. **11**: p. 546106.

Preperation of rVSV-GFP stock

Infection of Huh7 cells for new stock:

Huh7 cells bottles seeded the day before (1:3 of a >90% confluent bottle)
should be 90-100% confluent

- Mix 1 ml supernatant (aliquot) per bottle with 4 ml 0% DMEM++.
- Remove old medium.
- Add inoculum (approx. 5 ml) to the cells
- Incubate for 1 hour at 37°C, shaking occasionally
- After 1 hour, remove inoculum
- Add 10 ml of 3% DMEM++ to the cells
- Incubate for 24 hours

Harvest:

Very strong CPE, cells almost completely detached and strong GFP signal

- Transfer the supernatant to a 15 ml tube and centrifuge (10 min at 1500 rpm)
- Aliquot the supernatant and freeze it at -80°C.