Development of cell culture assays for identification of potential Zika virus inhibitors
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Abstract

Zika virus is a member of Flaviviridae family with 11 kbp long (+)ssRNA and consists of a single polyprotein that gets cleaved into 3 structural and 7 nonstructural proteins. The nonstructural protein 3 (NS3) is especially important in drug development due to its involvement in viral particle production and maturation. The N-terminal domain of NS3 harbours a catalytic triade that together with the cofactor NS2B functions as a protease. Since this protease is involved in cleavage of the polyprotein into mature, fully functional viral proteins, it represents an attractive drug target. In previous in silico studies performed by our research group we screened a library of 6265 compounds that showed some potential to work as NS2B/NS3 inhibitors. In order to study some of the compounds from that library that were also commercially available, there was a need for development and optimization of cell culture assays for Zika virus. Two assays were developed in this study: a plaque assay and an MTT based colorimetric assay. None of the three compounds displayed an inhibitory effect, but a functional positive control was found. AEBSF is a general irreversible serine protease inhibitor that in our study displayed an EC50 value of 110 µM with both MTT and plaque assay. Key points for future research are to test compounds that displayed better results in silico but are still not commercially available, to develop and optimize more cell culture assays for ZIKV and, most importantly, to find a good and safe vaccine.

Key words: ZIKV, inhibitor, NS2B/NS3, MTT, plaque assay, cell culture

Abbreviations:  
ZIKV                 Zika virus
DENV                Dengue virus
MTT                 (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
DC-SIGN             Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
Tyro3               Tyrosine-protein kinase receptor
AXL                 AXL receptor tyrosine kinase
AEBSF               A8456-4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
**Popular scientific summary**

Zika virus is a close relative of tick-borne encephalitis virus, dengue virus and yellow fever virus that has become very widely known in the past few years. The last outbreak happened in 2015 in Brazil where over a million were infected and around 5000 cases of fetal microcephaly appeared. There is still no available vaccine or other therapy available and research in this area is much needed.

In this study we tried to develop and optimize a cell culture assay that would enable the search for a Zika virus inhibitor. The experiments were performed on a cell line called Vero E6, which comes from a kidney of a monkey. The reason behind using this particular cell line is that we already know Zika virus can enter and infect them. Previous work done by our research group screened a library of around 6000 compounds that showed some potential to act as inhibitors on one of the Zika virus proteases. Three of out those were selected to be evaluated, despite the fact that they were not the best that could be obtained from computer simulations, but are commercially available. To test their activity against Zika virus, we developed and optimized two different cell culture assays: plaque assay and an MTT based colorimetric assay. A plaque assay is an old and well tested method, which allows observing the effects of a virus on cells with the naked eye. When the virus starts to replicate successfully, it causes damage to the cells shown as a white spot. The amount of such spots gives an idea of how much of the viral load is inside the cell line and how well it is infected by the virus. If the number of plaques reduces or disappears completely when adding a certain compound, one could assume that this compound might have an inhibitory effect on the virus. The MTT colorimetric assay is based on addition of two special reagents to the infected and treated cells and measurement of the absorbance of the reagents. The higher the value of absorbance, the higher the number of alive cells, and the lower the value of absorbance, the lower is the number of alive cells.

Unfortunately none of the mentioned compounds indicated inhibitory effects, but we managed to discover a useful positive control to test the reliability of our assays – a compound named A8456-4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). AEBSF is a small serine protease inhibitor that in our experiments displayed an EC$_{50}$ value (a concentration of a drug that shows 50% of its maximal effect) of approximately 110 µM. This study was a valuable first step towards finding compounds to treat Zika virus infection and it provides a solid ground for further research. Future goals are to develop more cell culture assays that would help to detect useful drugs, to synthesize and evaluate the activity of compounds with better results in computer simulations and ultimately to find a safe and effective vaccine.
1. Introduction

1.1. Zika virus: structure and genome

Zika virus (ZIKV) is classified into genus Flavivirus of Flaviviridae family, together with dengue virus, yellow fever virus, West Nile virus and TBE virus. Its genome consists of approximately 11 kbp long positive-sense linear ssRNA that encodes a single polyprotein of 3500 amino acids. The polyprotein is later cleaved by host and viral proteases into three structural (C, prM, E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). prM is cleaved by furin into mature pr and M so both can protect protein E during assembly. Protein C has a role in formation of the nucleocapsid while protein E is an envelope glycoprotein which exists in a homodimer form and is a target for neutralizing antibodies. Non-structural proteins are important for replication and cell-mediated immune response. The enveloped, icosahedral virion measures about 50 nm in diameter and has T=3 organization of surface dimers. The prM protein gets cleaved upon conformational change in the viral membrane due to low pH. It is 42% identical to prM in dengue virus – some residues in both variants of the same protein are highly conserved and despite the many similarities those two viruses share, the required pH drop for prM cleavage seems to be a trait of ZIKV only. Env glycoprotein is a surface homodimer, consisted of three β-sheets. It is approximately 504 amino acid residues long and shows similarities to DENV3 Env protein (~ 58% identity). Nonstructural protein 1 is another protein that has a role in replication. In mammals, replication occurs in the cytoplasm (surface of endoplasmic reticulum). Zika enters the cells by attaching its E protein to one of the 3 host cell receptors: DC-SIGN, Tyro3 or AXL. During replication in the cytoplasm, dsRNA is formed from genomic (+)ssRNA, which gets transcribed and replicated in order to manufacture more genomic (+)ssRNA and mRNA. After assembly of new virions and budding, they exit the cell by exocytosis. The NS3 protein, which is involved in processing of the polypeptide chain and genomic replication, consists of a N-terminal protease domain and a C-terminal helicase domain. The C-end with its nucleoside triphosphatase activity takes care of the energy needs for RNA intermediates and promotes genome replication with the help of RNA-dependent RNA polymerase.

1.2. History, epidemiology and pathology

The virus was first isolated from a febrile rhesus monkey in Zika forest (Uganda) in 1947. After that it was isolated from A. africanus mosquito species in 1948 and it was believed that infection with Zika virus is a zoonose, transmitted by Aedes mosquitoes. The first evidence of human infection was obtained in Nigeria in 1968 and in the time period of 1971-1975 when they conducted a study where 40% of tested subjects were seropositive for neutralising antibodies to Zika virus. The infection is usually asymptomatic (around 80% of cases); in opposite case the incubation period is 3-12 days followed by mild, self-limiting symptoms that resemble other arbovirus infections. Those symptoms include fever, rash, joint and muscle pain, headache and
conjunctivitis. However, during the outbreak in Brazil there were a significantly higher number of reported cases of microcephaly in newborns. Therefore it was suggested that Zika virus could be responsible for fetal abnormalities besides flu-like symptoms. Another suggested complication that might be connected to Zika virus infection is Guillain–Barré syndrome in adults – an autoimmune disease resulting in muscle weakness due to immune-damaged neurones. Besides vector transmission of this virus, there are other routes such as sexual, congenital and perinatal. At the site of inoculation, virus replicates first in keratinocytes and dendritic cells, until it migrates to the bloodstream and the lymphatic system.

1.3. Protease inhibitors as potential drugs

Proteases are divided into 7 different groups based on their catalytic properties: cysteine, serine, aspartic, threonine, metallo, glutamic and asparagine-peptidase. They have an important role in protein function and consequently take part in many cellular processes, such as cellular signalization, growth, differentiation, and immune response, which makes them suitable drug targets. In order for them to be useful in antiviral therapy, certain criteria must be met. Besides the potency of a drug, it is desirable for a protease inhibitor to have high specificity in binding and optimal pharmacokinetical and pharmacodynamical characteristics. It is also important that the inhibitor is a stable compound, can be administrated orally and with minimal adverse side effects. The traditional approach to finding protease inhibitors is product screening for lead compounds and optimization. Such screening has been improved in the last couple of years with various 3D methods and other tools in bioinformatics. Protease inhibitors are used as therapeutic agents in treatment of HIV, HCV and herpesvirus infections (CMV, HSV-1, HSV-2).

1.4. Flaviviral NS2B/NS3 protease

NS2B/NS3 protease is a viral serine protease that ensures cleaving the polyprotein into mature, fully functional viral proteins and therefore it represents a potential drug target. N-domain of NS3 protein is a serine protease and C-end is a NTPase-helicase. In order for the serine protease to function, it requires the presence of its cofactor NS2B. NS2B is an integral membrane protein, consisted of three domains: central domain serves as a cofactor for NS3 protease and it is surrounded by a transmembrane segment at both ends. Without the central part of NS2B, NS3 is insoluble, unable to perform its catalytical activities and most likely can not fold properly. Its N-end is hydrophilic and induces solubility in NS3, while C-end of the central region makes a β-hairpin that shapes S2 nd S3 in binding pockets of NS3 and with that directly interacts with the substrate. Cleavage of the NS2B/NS3 site occurs in cis or trans conformation and it is similar for all flaviviruses, including dengue, Zika and yellow fever virus.

1.5. Dengue virus protease

Dengue virus due to being another member of genus Flavivirus, shares a lot of common characteristics with ZIKV including Aedes mosquitoes as vectors and molecular properties. It measures 50 nm in diameter and encodes a 10,732 bp RNA, which yields a polyprotein of a similar length as ZIKV. There are four known serotypes of the disease (dengue fever or dengue hemorrhagic fever) – DENV1-4 with different levels of symptom severity. NS3 protein besides serving as a protease for polyprotein cleaving with NS2B as its cofactor, also performs capping of nascent viral RNA and unwinding of dsRNA (replicative state). The protease function is crucial in infecting host cells and viral replication. Cleavage site of dengue virus NS2B/NS3 protease is represented by a catalytic triade of His51, Asp75 and Ser135 and it cleaves several sites besides NS2B-NS3; NS2A-NS2B, NS3-NS4A and NS4B-NS5. Overall treatment of infection of dengue virus includes targeting NS3 and NS5 proteins, which has a successful
outcome. NS5 protein is the largest and has dual purpose: has a N-terminal methyltransferase domain (Mtase) and C-terminal RNA-dependent RNA polymerase (RdRp). Antiviral agents inhibit either virus entry, its capsid, helicase, protease, polymerase, methyltransferase, NS4B or even host targets.\textsuperscript{23}

1.6. HIV protease
Human immunodeficiency virus (HIV) is a retrovirus that causes acquired immunodeficiency syndrome (AIDS) and development of antiviral agents against has been an important field of research for many years.\textsuperscript{19,24} Firstly these agents were nucleoside analogues that targeted reverse transcriptase, followed by non-nucleoside analogues, but since both showed certain limitations in treatment such as low success rate, adverse side effects and emergence of resistant strains, there was a need for an further combination treatment.\textsuperscript{19} A new target was a HIV protease since this target is also crucial for the virus replication.\textsuperscript{25} HIV-1 protease is an aspartic acid protease in a homodimer form that resembles human aspartic acid proteases (e.g. pepsin, cathepsin D) and it is translated from gag-pol polyprotein.\textsuperscript{26} The active site is consisted of two Asp-Thr-Gly sequences and the regions that cover the active site participate in biding of substrates or inhibitors.\textsuperscript{19} Some of the clinically approved HIV-1 protease inhibitors are saquinavir, nelfinavir, indinavir and ritonavir.\textsuperscript{19} Their general mechanism of action is interfering with maturation of newly formed virions by preventing the cleavage of gag and gag-pol protein precursors. Therefore these drugs prevent further infections and have virtually no side effects.\textsuperscript{26,27}

1.7. HCV protease
Hepatitis C virus is a major cause of chronic liver disease and belongs to the Flaviviridae family and genus Hepacivirus.\textsuperscript{28,29} Since it belongs to the same family as Zika and dengue virus, a certain analogy can be observed in structure – its nonstructural proteins are named NS2, NS3, NS4 and NS5.\textsuperscript{30} NS3 protease is the most common target in antiviral therapy due to its role in viral replication.\textsuperscript{29} As all other flaviviruses, HCV has conserved residues His-Asp-Ser in its N-domain of NS3 which resemble the catalytic triad of serine protease and therefore indicate involvement of NS3 in polyprotein processing.\textsuperscript{30} The serine protease encoded in NS3 cleaves sites NS3-4a, NS4a-NS4b, NS4b-5a and NS5a-b with the help of cofactor NS4a, while junction at the NS2 region requires another protease in order to be cleaved – it consists of NS3 protease domain and most of the NS2 domain (although the actual catalytic activity of NS3 is not required).\textsuperscript{31–37} In contrast to flaviviruses, HCV's NS4A cofactor only has a structural role and no direct catalytical abilities like NS2B.\textsuperscript{21} Widely used antiviral protease inhibitors for treatment of patients, infected with HCV are inhibitors of NS3/4A serine protease such as simeprevir, paritaprevir together with ritonavir, and grazoprevir. Other classes of inhibitors used in combination treatment are NS5B polymerase inhibitors, for example sofosbuvir and NS5A inhibitors, such as daclatasvir and ledipasvir.\textsuperscript{38–40}

1.8. Molecular docking and molecular dynamics simulation
Molecular docking is an approach which allows modelling of interactions between a protein and a small molecule at an atomic level. This method consists of two steps: first one is ligand conformation, position and orientation prediction and the second one is assessment of binding affinity. The results display the behavior of small molecules in binding sites of proteins which is very useful in the search for new, specific drugs.\textsuperscript{41} Molecular dynamics simulation relies on two methods: energy minimization and and molecular dynamics.\textsuperscript{42} Both of the mentioned methods allow structure optimization and simulation of natural motion of biological molecules,
respectively. With obtained results it is possible to understand structure-to-function relationships between observed molecules.\textsuperscript{43}
2. Aim

The aim of this project was to develop cell culture assays for Zika virus that could be used for the identification of a potential inhibitory role of compounds X, Y and Z on NS2B/NS3 protease. Our research group has previously gathered in silico data (unpublished results) in screening a library of 6265 compounds that could work as ZIKV protease inhibitors. Three of those compounds were selected for their activity to be tested in vitro – compounds X, Y and Z. Although these did not display the best results in molecular docking and dynamics simulation, they are still the best ones of those commercially available.
3. Materials and methods

3.1. Cell culturing
For this study was used an immortal cell line Vero E6, derived from the kidney of an African green monkey (Cercopithecus aethiops) in the 1960’s. This cell line is also known as VeroC1008 and it is a clone from Vero76. Pre-prepared frozen stock from the nitrogen tank was thawed and cultured on 25 cm² and 75 cm² tissue culture flasks with flat bottom and vented caps. As growth medium was used DMEM containing phenol red with added FCS (5%), L-glutamine and PenStrep. Cells were incubated in Heracell™ incubator at 37 °C and 5% CO₂. Passaging was needed every 24-48 h or when the cells reached ~90% confluency.

3.2. Plaque assay
Experiments were performed by infecting confluent Vero E6 cells cultured on 6 well Corning® Costar® cell culture plates with flat, transparent bottom. Pre-prepared frozen stock of Zika virus MR766 strain, which was kindly provided by Åke Lundkvist, was thawed and 10-fold dilutions were prepared in DMEM (containing 1% FCS, L-glutamine, 1% PenStrep, and phenol red). The assay was performed as follows: Vero E6 cells were seeded in wells by adding 1 ml of cells with the concentration of 6.5 x 10⁵ cells. After 24 h of incubation the cell monolayer was confluent and ready to be infected. The medium from wells was discarded and cells were washed with PBS before infection with 500 µl of different virus dilutions. After the infection, the cells were incubated for an hour with gentle rocking of plates every 15-20 min. After 1 h of incubation time, the remaining supernatant was discarded and the cells were washed again with PBS. For titration of viral stocks, the monolayers were covered with an agar overlay without any PI inhibitor. When performing plaque reduction assay, an agar overlay containing different concentration of PI was added to each well. The agar overlay consisted of 0.5% Noble agar, MEM (2X), FCS (1%), PenStrep and the selected compound to be tested. Upon agar solidification, plates were incubated at 37 °C, 5% CO₂ for 5 days before staining with Neutral Red dye at a concentration of 50 µg/ml.

3.3. MTT based colorimetric assay
A Cell Proliferation Kit I (MTT) (Sigma-Aldrich) was used to identify the quantity of virus that kills a certain amount of cells in 4 days post infection and to quantify the difference in fluorescence between cells treated with compounds X, Y and Z versus the nontreated. The general function of this assay is the cleavage of yellow-coloured tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) in a metabolically active cell, where NADP and NADPH are involved. Solubilized formazan crystals yield a coloured solution that can be quantified with an ELISA reader. The measurements were performed in an ELISA reader at a wavelength of 590 nm or 490 nm 24 h after the addition of solubilization solution. Collected data was exported to Excel and later to GraphPad, where it was analyzed and visualized. This method was used in several different ways to quantify the potential inhibitory effect of three different compounds (X, Y and Z). Vero E6 cells were cultured in Corning® CellBIND® 96 well plates by seeding 50 µl of 2 x 10⁵ cells/ml (10,000 cells in each well). The cells were then incubated in DMEM supplemented with 5% FCS for 24 h. After the incubation time, the virus stock was 5-fold serially diluted directly into the 96 wells plate. The serial dilution of the concentration of virus that killed more than ~60% of the cells in 4 days was used in further experiments. The addition of compounds X, Y, or Z (Sigma-Aldrich) accrued after 1 h post infection or simultaneously with the infection. The virus stock was diluted in DMEM with 1% FCS without phenol red to exclude any interference in the measurement of absorbance. The
different compound were serially diluted and 25 µl of the desired concentration (X4) was added to the respective wells to a final volume of 100 µl and 1x concentration. The compound A8456-4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (or AEBSF, Sigma-Aldrich) was used as positive control.

3.3.1. CCID determination with the MTT assay
The MTT assay was also used to find an appropriate concentration of the virus that would infect the cells in a desired time span. 50 µl of cells with the concentration of 2 x 10^5 cells/ml (10.000 cells in each well) were seeded into a 96 well plate. After 24 h of incubation 50 µl of DMEM without phenol red was added into each well. Infection with the virus was performed in 5-fold serial dilutions by adding 25 µl of viral stock into the first well and then moving to neighbouring wells, adding the same amount of the viral dilution from previous well to the next well. 4 days post-infection the MTT assay was performed to find the desired (~ 60%) CCID value.
4. Results

This project was focused on developing a cell culture assay for Zika virus which would allow investigation of potential NS2B/NS3 inhibitors. Compounds X, Y and Z were chosen to be evaluated *in vitro* due to their score in molecular docking and their commercial availability. Compound X did not display promising results in neither molecular docking or dynamics simulation, hence testing its activity *in vitro* was done merely to support the *in silico* obtained data. Compound Y was one of the 25 best compounds that displayed good results in molecular docking, but performed poorly in dynamics simulation. Due to its good docking results, it was selected for further, *in vitro* investigation. Compound Z showed good results in both molecular docking and dynamics simulation, therefore it was a reasonable choice for *in vitro* studies.

To obtain *in vitro* results, two assay systems were established – plaque assay (together with plaque reduction assay) and an MTT based colorimetric assay. Plaque assay would allow observation of difference in number of formed plaques in cells which were infected with Zika virus and either treated with the selected compound or not. Cells which were not infected with the virus, but still treated with a selected compound, were an indication of possible toxicity effect of the compound. On the other hand, MTT assay enabled collecting quantitative data in the form of measuring absorbance. The setup was the same as in plaque assay – infected/treated, uninfected/treated, infected/untreated and uninfected/untreated cells were used in each experiment.

4.1. MTT colorimetric assay

The MTT cell viability assay was used to both quantitatively and qualitatively evaluate the activity of compounds X, Y, Z as well as to evaluate the suitability of this assay by using the compound AEBSF as positive control. This assay is based on cleavage of yellow-coloured tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) in a metabolically active cell. After addition of both reagents to the cells, the measurement of absorbance was possible. The higher the quantity of alive cells in the wells, the higher is the value of absorbance. The general concept of performing this type of experiment was to either infect the confluent wells in 96 well plate first and treat them with a selected compound after 1 h of incubation or infect the cells with the virus and treat with a target compound at the same time (both variants gave the same results (data not shown)). Treatment of cells occurred in noninfected cells as well to investigate the potential toxicity of the candidate drugs. The actual usage of MTT kit happened 4 days post infection and treatment. The first reagent was added into each well that contained the target compound (infected or noninfected) together with controls (either uninfected and untreated cells or infected and untreated cells). The first MTT reagent was incubated for 4 hours before the second reagent was added into the same wells as the first one. The cells were ready for measurement in an ELISA reader after 24 h incubation of the second MTT reagent. This assay offered both quantitative and qualitative data; besides collecting measurements in a form of absorbance of content of the wells at 590 nm, rough estimation of viability of the cells could be done by simply looking at the shade of colour in the wells. Yellow shades indicated that the viability of the cells is low while purple shades suggested the viability is high.

4.1.1. Compound X

Vero cells in 96-well plates were infected with 4X CCID$_{60}$ concentration and treated with a range of concentrations that spanned from 100 µM to 100 nM of compound X 1 h post infection.
This experiment was designed to determine if the presence of compound X in infected cells shows any difference in viability when comparing to infected/untreated cells, as well as noninfected/treated cells. Although this compound did not show good results neither in molecular docking or simulations, it was still tested and interpreted as the negative control in the assay. Figure 1 displays the normalized percentage of alive cells when treated with different concentrations of compound X. The bar that represents noninfected/treated cells at 100 µM suggests toxicity effect (the value is far below controls). This effect is not observed in other concentrations since all the other bars from the same category are in the same range as the orange control. To observe inhibitory of this compound, the green bars (infected/treated cells) should be approximately in the same range as either noninfected/untreated control or the noninfected/treated cells. While this is not the case in Figure 1, the compound X does not display inhibitory effect on ZIKV infected cells.

![MTT assay inhibitory profile of compound X](image)

**Figure 1.** The normalized percentage of alive cells when infected and treated with different concentrations of the compound X and the controls.

### 4.1.2. Compound Y

Further investigation in this project was focused on compound Y and its effect on ZIKV in Vero E6 cells. It included addition of the compound Y in separate wells while simultaneously infecting with a CCID₇₀ concentration of the virus. Infected/untreated and noninfected/untreated cells represented the controls. The absorbance from noninfected/treated cells was compared the noninfected/nontreated control determine toxicity effect of the drug. Figure 2 shows the normalized percentage of alive cells when treated with different concentrations of compound Y. The values of noninfected/treated cells are in the same range as noninfected/untreated control which indicates the compound induces no toxicity effect in cells. The percentage of viable cells in infected/treated cells fall in approximately the same range of values as infected/untreated control, therefore the compound does not indicate any inhibitory effect on ZIKV infected cells.
Figure 2 shows the normalized percentage of alive cells when infected and treated with different concentrations of the compound Y. Because the green bars that represent infected/treated cells had almost the same value as the infected/untreated control, there was no inhibition.

4.1.3. Compound Z
The last investigated drug and its effect on ZIKV protease was compound Z. The cells were infected with CCID$_{60}$ concentration of the virus and treated with a wide range of concentrations of compound Z at the same time. Noninfected/untreated cells and infected/untreated cells controls, respectively, represented the references for 0% and 100% viability. The absorbance was measured at 490 nm.
Figure 3. The normalized percentage of alive cells when infected and treated with different concentrations of the compound Z and the controls.

Figure 4. The normalized percentage of alive cells when infected and treated with different concentrations of the compound Z and the controls.

Figure 3 and Figure 4 show the percentage of viable cells when infected with ZIKV and treated with compound Z in a concentration range of 2 µM to 100 µM together with the controls (see legend). Figure 3 shows that both infected/treated and noninfected/treated have approximately same values which shows toxicity of this compound at higher concentrations and no inhibitory effect (orange bars are much lower than the noninfected/untreated control). On the other hand,
Figure 4 shows the rise in absorbance value of both noninfected/treated and infected/treated cells at concentrations 6 µM to 2 µM. The error bars in those series are either low or not shown due to small number of measurements, therefore it is difficult to make any conclusions on compound Z’s effect on ZIKV NS2N/NS3 protease.

4.1.4. A8456-4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)
Besides testing the activity of compounds X, Y and Z, there was also a need to discover a working positive control for the assays. For this purpose a general irreversible serine protease inhibitor A8456-4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) was used. AEBSF is an irreversible, cell membrane permeable, serine protease inhibitor capable of successful inhibition of trypsin, chymotrypsin, plasmin kallikrein and thrombin while showing low toxicity, good solubility in water and good stability in aqueous liquids. Quantification of its activity was made identically as for compound Z; infection with the virus simultaneously with treatment, incubation for 4 days, addition of both MTT reagents and measurement with ELISA reader at 490 nm. Figure 5 represents the normalized percentage of cells, infected with ZIKV and treated with AEBSF concentrations in the range of 1 µM to 300 µM. The slope of the curve displays an inhibitory activity of AEBSF with an EC50 value of approximately 110 µM.

![MTT assay inhibitory profile of the positive control](image)

**Figure 5.** The normalized percentage of alive cells when infected and treated with different concentrations of the positive control AEBSF.

4.1.5. Cytotoxic effect of DMSO on Vero E6
To exclude any possibility of cytotoxic effect due to dilution of compound X in DMSO, Vero E6 cells were seeded in a 96 well plate the same as in all other MTT experiments and incubated with DMSO for 4 days before addition of both MTT reagents and measurement. Figure 6 displays the obtained results of absorbance of cells that were containing DMSO vs. those without. The bars that represent cells containing different concentrations of DMSO are slightly
lower than those without DMSO. Approximately 20% more viable cells were detected in controls than in cells with DMSO. To conclude if DMSO really has a cytotoxic effect on this cell line, more experiments should be performed since the colour of the wells was purple in both controls and cells with DMSO (data not shown).

**Figure 6.** Comparison of absorbance of cells containing different concentrations of DMSO versus controls (cells with only growth medium).

### 4.2. Plaque assay

Several plaque assays and plaque reduction assays were performed to titrate viral stocks, optimize the assays and access the correct functioning of the assays. The correct functioning of the plaque reduction assay was accessed by using a general irreversible serine protease inhibitor A8456-4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) as a positive control. Once the assays were functional and optimized, the inhibitory profile of compounds X, Y and Z were again investigated and compared to previous *in silico* results. The general steps for the plaque assays were (in order): 1) seeding of Vero E6 cells and incubation for 24 h, 2) confluent cells washed with PBS and infected for 1 h, 3) virus inoculum discarded and cell monolayer washed with PBS again, 4) addition of agar overlay (with or without the selected compound), 5) staining of cells with Neutral Red dye five or eight days after infection for increasing the contrast and visualizing the plaques.

#### 4.2.1. Plaque assay optimization

The optimization of the plaque assay consisted of a series of experiments that aimed to identify the 1) the right amount of viral inoculum for cell infections, 2) the best concentration of fetal calf serum (FCS), 3) volume of overlays and 4) the right amount of Neutral Red dye. The concentration of FCS used for the viral dilution/infection and FCS concentration supplemented in the agar overlay were selected to minimize the degradation of the confluent cell monolayer, which could interfere with plaque formation and result interpretation. The ideal concentration of FCS was determined as follows: one day prior to infection 1.2 x 10⁵ cells/ml were seeded into a 24 well plate and incubated overnight. After incubation, the cells were confluent and ready to be infected. For the infection, the virus was serially diluted in DMEM supplemented
with 0, 1 and 5% FCS, respectively. The cells were infected in triplicates with 200 µl of virus inoculum for 1 h, as previously explained. Controls, also in triplicates, were represented by noninfected cells, cultured in 500 µl of DMEM containing either 0, 1 or 5% FCS. The plates were incubated for 8 days and the state of the cell monolayer was monitored every day. From the assay results it appeared that 0% FCS had no negative effect on control (uninfected cell monolayer) with limited monolayer degradation 6 days post infection, while the dilution of virus in DMEM containing no FCS completely abrogated the cell layer (Table 1). Accordingly, 1% FCS caused only limited negative effect on the cell monolayer health after 6 days of incubation, while a clear sign of infection, such as vacuolation, was clearly visible one day post infection (virus dilution: 1 x 10⁻¹). Using 5% FCS did not prevent cell infection but caused a limited cell degradation as soon as 3 days post infection which progressed for the whole duration of the experiment. Given the results, it was clear that 1% FCS was the best concentration to be used in further experiments.

<table>
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<tr>
<th>FCS %</th>
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<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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Table 1. Vero E6 monolayer degradation dependent on FCS concentration in growth medium. Legend: - No monolayer degradation, + very limited degradation, ++ limited degradation, +++ extended monolayer degradation

Besides concentration of FCS in DMEM medium, the volume of the virus had an impact on cell layer damage. The viral inoculum was optimized by selecting different volumes of DMEM for the mock infection of cell monolayers. For the 6 well plate the volumes ranged from 250 µl to 500 µl. Once the cells were mock infected, they were placed in an incubator for 1 h. Then the inoculum was discarded and 3 ml of DMEM was added in each well. After 24 h incubation the monolayer could be observed in all inoculum concentrations, except the 500 µl (see Figure 7). Figure 7 displays the outcome: white areas represent cell damage and the blue areas were cells that seemed intact. All volumes under 500 µl were inadequate and caused cell layer damage (white spots inside circles, Figure 7).

![Figure 7](image_url)
The time of incubation post infection was chosen to allow countable plaque formation avoiding the merging of plaques and misscalculation of the viral plaque forming unit during virus stock titration. The length of incubation after agar overlay addition was determined by staining the cells after 8 days or 5 days after incubation. After 8 days the plaques were too large for counting and were colliding with each other while after 5 days the plaques were large enough to be counted and at the same time small enough not to collide with each other.

The right volume of overlay and Neutral Red dye was optimized as follows: Neutral Red dye was diluted to different concentrations and added either into the first and only overlay or it was a part of the second overlay. The first and only overlay was added to the cells 1h post-infection and staining happened 5 days after that (on top of the agar), while in the case of using two overlays, the first one was added 1h post-infection and the second one (which harboured the dye) after 5 days. The shade of the colour in wells after staining allowed clear distinguishment of plaques and determination of the appropriate concentration of the dye.
4.2.2. Plaque reduction assay
A plaque reduction assay is the gold standard method that allows testing of antiviral susceptibility. In this study the antiviral susceptibility for the compound AEBSF was investigated. 47

Figure 8. Plaque assay of the positive control AEBSF. Top and bottom left wells contain infected cells treated with 100 µM AEBSF. Top and bottom well on the right show wells of uninfected cells, treated with 100 µM AEBSF.

Figure 8 shows the plaque assay of the positive control AEBSF with a concentration of 100 µM. In both wells on the left side there is plaque formation visible and the wells on the right were used as an noninfected control.
Figure 9. Plaque assay of the positive control AEBSF. Top and bottom left wells contain infected cells treated with 200 µM AEBSF. Top and bottom wells in the middle show uninfected cells, treated with 100 µM AEBSF. Upper right well: uninfected, untreated cells.

Figure 9 shows the same assay as Figure 8, but with a higher concentration of AEBSF. Both wells on the left where there were infected cells and treated with 200 µM AEBSF, the middle wells were uninfected and treated with the same concentration of AEBSF and on the upper right is the uninfected and untreated control. In this case there was no plaque formation in the infected wells. Furthermore, when treating infected cells with 10 µM of AEBSF, all cells died. When infecting and not treating the cells, plaques in a higher quantity were formed than in Figure 8 (data not shown).
5. Discussion

There are currently no available treatment therapies or vaccines for ZIKV infection, which makes this area an interesting novel field of research. It raises the need for screening of lead compounds that could work as drugs with a successful outcome in therapy or be used as a prophylaxis. In this study we aimed to develop and optimize cell culture assays for identification of compounds that could act as ZIKV NS2B/NS3 protease inhibitors. Around 6265 already existing compounds were screened against ZIKV NS2B/NS3 protease (unpublished data) and the molecular docking was performed by using AutoDock Vina and iGEMDOCK programs. From the molecular docking results several compounds displayed potential binding affinity towards ZIKV NS2B-NS3 protease. The ones that showed the best results in both docking and simulations are still not commercially available. Therefore, for our experiments we used three compounds from the screened library that did not show the best results, but were commercially available. Compounds X, Y and Z were investigated to determine their actual activity in vitro until the compounds that displayed better results in docking will become available and to compare the obtain in silico results with in vitro results. Compound X performed poorly in both docking and simulation and its activity in vitro was tested to prove the in silico results. On the other hand, compound Y performed slightly better than X, yet still not exceptionally well. Since it was already accessible in our laboratory, it was tested in vitro. Compound Z showed good results in docking and simulations, therefore it was the most promising molecule to be investigated in vitro.

A recent publication described a similar approach in finding a ZIKV protease inhibitor. From a high throughput screen of 40,000 compounds they previously identified several HCV NS3/NS4A inhibitors with low IC\textsubscript{50} values. Some of them are also selective for NS3 protease, therefore they tried to investigate if any of them can inhibit ZIKV NS2B/NS3 protease. As the active sites of HCV PI are very shallow, flexible and solvent exposed, it is difficult to repurpose them for treatment of other viral infections, such as ZIKV. One successful inhibitor reported so far is a capped boronic acid compound cn-716 that reversibly inhibits ZIKV NS2B/NS3\textsuperscript{pro} with an IC\textsubscript{50} value of 0.25 ± 0.02 μM and K\textsubscript{i} = 0.040 ± 0.006 mM.\textsuperscript{20}

The search for a ZIKV NS2B/NS3 protease inhibitors has so far lead to development of mainly enzymatic and only a few cell culture assays, and therefore it is difficult to find good comparison for the data we have obtained. A close approximation would be assays of dengue virus or West Nile virus, since both are ZIKV relatives from the same Flaviviridae family. Kuiper et al. described an enzymatic assay that involved the measurement of either unlinked (containing a cleavage site at the junction of NS2B) or linked (artificial or natural) ZIKV NS2B/NS3 activity. In their study there was no cleavage site between NS2B and NS3 in the presence of a compound A which is supposed to be a known DENV and WNV inhibitor. Their data displayed activity of the unlinked protease to be 6-fold more active than linked protease which persisted the entire time in the two hour long incubation. However, their compound A inhibited both linked and unlinked protease with similar potency, and therefore it is difficult to conclude that this compound truly possesses inhibitory effects as they expected.\textsuperscript{48}

Our MTT assay was adapted from Han et al. publication where they investigated the role of resveratrol agents as potential anti-dengue agents in cell culture assays.\textsuperscript{49} Their study included usage of 26 resveratrol agents (3,5,4'-trihydroxy-trans-stilbene) which are synthetic derivatives of a natural compounds found in many fruits and vegetables, mostly grape skin and peanuts. The reason behind selecting these compounds was a study conducted earlier that displayed anti-HIV and anti-cancer activity.\textsuperscript{49,50} The mentioned 26 analogs were tested to investigate possible inhibitory effect on HIV with a MTT assay. Two of the analogs showed a dose-dependent
inhibition with EC$_{50}$ values of 8.12 ± 0.82 nM and 7.22 ± 0.85 nM, respectively. As positive control was used a compound called MPA, an inhibitor of dengue virus replication that displayed an EC$_{50}$ value of 0.96 µM. Compared with our collected data, EC$_{50}$ values in their case were much lower in both tested compounds and the positive control. Their study was supported with cytotoxicity assay that included addition of DMSO to their Huh-7 cell culture which our study included as well, but on Vero E6 cells. Another approach in investigation of resveratrol agents activity against DENV-2 was by a qRT-PCR experiment that detected the RNA levels in their samples which might be a reasonable supportive method in our study as well in the future.

Since compound X at 100 µM displayed a toxicity effect (Figure 1) and did not indicate any inhibitory role on ZIKV infected cells at lower concentrations, we can conclude that it is not suitable for further research concerning possible treatment agents. This supported in silico results where this compound X did not perform well in either docking or simulations. Furthermore, we also tested the possible involvement of different concentrations of DMSO on cell toxicity which resulted in only 20% less viable cells when DMSO was present (see Figure 6). To find the real reason for this toxicity, more experiments should be done since this compound is a part of a treatment of another disease and it is most likely not toxic at our selected concentration range.

On the other hand, compound Y displayed no toxicity effect in any of the concentration we used for treatment of cells (Figure 2). Since the bars in infected and treated cells were in an approximately the same range as infected/untreated controls, the inhibitory role was disproved.

Measurement of absorbance of compound Z showed slightly different results as previously tested compounds. It seems as if higher concentrations (from 100 µM to 7 µM) have no inhibitory effect at all and maybe even induce toxicity in the cells, while lower concentrations (from 6 µM to 2 µM) seemed to show higher amounts of viable cells in both infected/treated as well as uninfected/untreated cells. The error bars for these measurements were quite high and there might be several explanations for this variability, hence it is not possible to neither confirm or disprove the potential inhibitory effect of this compound. Besides, in silico data did not suggest this compound to have a great potential for inhibiting the NS2B/NS3 protease. In order to get completely reliable information about compound Z's effect on ZIKV in Vero E6, we would need to perform many more experiments both with the MTT assay and plaque assay.

One of recently published articles described development of a simple, fast and cheap colorimetric assay for ZIKV on the same type of cell line and the same MR766 virus strain that we used in our study.51 A few details in the structure of their experiment differed from ours, but overall the concept was the same: incubation of Vero E6 cells, infection with ZIKV, addition of both MTT solutions and measurement of the absorbance with ELISA reader at 490 and 630 nm. To support their findings with the MTT assay and to investigate the virus-induced CPE, a plaque assay was performed. CPE was observed in virus dilutions from 10$^{-1}$ to 10$^{-5}$ with visible plaques, but not in concentrations lower than 10$^{-5}$. Furthermore, they compared the MTT assay with an enzyme-linked CellTiter-Glo® Luminescent Cell Viability Assay to quantify the infectivity and both displayed similar results – the higher the concentration of the virus, the lower the amount of alive cells in observed wells.51

The plaque assay that we performed with AEBSF supported the EC$_{50}$ value results measured with the MTT assay. Figure 8 shows the presence of plaques in wells at the concentration of 100 µM while at 200 µM (Figure 9) they had a homogenous surface and no sign of plaque
formation. Successful inhibitory effect was expected due to its general mechanism mentioned before – irreversible inhibition of serine proteases. With our collected data we can confirm that this compound AEBSF is a suitable compound as a positive control in further research. There is a large variety of publications that describe AEBSF’s inhibitory effects; one of the earliest is from 1995.\textsuperscript{52} The report described an in vitro assay for HCV NS3 serine proteinase activity where they used AEBSF as a positive control. It inhibited both NS3 and NS3-4A proteinases at milimolar concentrations which is at an even lower concentration range than used in our experiments.\textsuperscript{52} In a more recent study a plaque reduction assay was performed with ZIKV and compound bromocriptine which reached nearly 100\% plaque reduction at a concentration lower than 10 \textmu M which is a 10-times lower concentration than the EC\textsubscript{50} value of AEBSF in our experiments.\textsuperscript{53}

An advantage of using plaque assay and a MTT assay to investigate the activity of our selected compounds is before everything else the simplicity of the methods, and both of them provided results in a reasonably short time and at a low cost. The plaque assay makes virus titration and general observation of the wells with infected cells a lot easier since once plaques formed they were visible both under the microscope and with the naked eye.

To discover a drug that would be a good therapeutic agent or used as a prophylaxis will require further research with more compounds and more emphasis on development of appropriate cell culture assays that would allow screening of lead compounds. Furthermore, there is still no available vaccine that would protect the population in the areas of disease and travellers to these parts against ZIKV infection. Only with a combination of a safe vaccine and an effective drug a complete eradication of the disease would be possible.
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