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New potential antivirals against tick-borne encephalitis virus infection

Bachelor thesis

Barbora Veselková

Supervisor: doc. RNDr. Daniel Růžek, Ph.D.

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BACHELOR THESIS

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ANOTATION

The experimental work of the thesis was concentrated on studying of the inhibition effect of twenty-six chemical substances on tick-borne encephalitis virus. The substances showing inhibition effect in High Throughput Screening assay were processed to Cytotoxicity assay, where their toxic effect on tissue cell cultures was determined. Subsequently, substances with inhibition effect and no cytotoxic effect were incubated with cell culture and virus in two different incubation time periods and the supernatant containing virions was examined using plaque assay.

AFFIRMATION

I hereby declare, that the Bachelor thesis was written solely by myself using documents and literature listed in the reference section.

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In Linz, 9. 5. 2013 ____________________________

Barbora Veselková

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1. INTRODUCTION

Tick-borne encephalitis vírus is an agent which causes tick-borne encephalitis, one of the most dangerous infection of human central nervous system which appears in Europe and Asia. This type of encephalitis is reported in 11 000 cases in Russia and in 3000 cases in Europe anually [1]. Percentage of a development of chronical problems, total treatment from the illness and death varies with the tick-borne encephalitis virus strain (but also other factors are involved). However, there are no effective antivirotics existing up to date, there are various types of vaccines agains this virus available on the market.

1.1. Classification

The tick-borne encephalitis virus belongs to the family *Flaviviridae* and genus *Flavivirus,* which contains 53 species of viruses [2] and is divided into 3 clusters: arthropod cluster containing tick-borne and mosquito-borne cluster and "no known arthropod vector" cluster. Flaviviruses are arthropod-borne viruses (arboviruses), what means that the virus is transmitted to the final host in saliva of an arthropod vector. Mosquito-borne cluster contains 7 groups of viruses (e.g. Japanese encephalitis, Dengue or Yellow fever virus group) which are transported to human body in the saliva of mosquitoes. No known arthropod vector cluster is divided into 3 groups: Entebbe, Modoc and Rio Bravo virus group, but the arthropod vector is unknown. The last one – tick-borne encephalitis cluster contains 2 groups: mammalian and seabird virus group, which are transmitted to the final host in the saliva of ticks. Mammalian tick-borne virus group contains viruses which affect humans, for example, Omsk hemorrhagic fever, Louping ill virus or the one mentioned before – Tick-borne encephalitis virus [3]. Moreover, the tick-borne encephalitis virus is divided into three subtypes: Far Eastern (previously known as Russian Spring/Summer encephalitis virus), Siberian (previously West-Siberian encephalitis virus) and Western European virus subtype (previously Central European encephalitis virus) [1].

1.2. Molecular structure of Tick-borne encephalitis virus and function of its individual parts in virus life cycle

Mature virions of the Tick-borne encephalitis have 50 nm in diameter and are formed by lipid bilayer, which surrounds the core (Fig. 1(A)). The bilayer consists of two structural envelope glycoproteins: E (envelope) and M (membrane). Immature virions contain precursor glycoprotein prM, which changes to the M glycoprotein when the virion exits the host cell [4]. The function of E glycoprotein is to recognize the cell receptor and mediates the fusion of the virus with the membrane of the endosome [5]. Molecular weight of E glycoprotein is 50 – 56 kDa and of M glycoprotein 7 – 9 kDa. The core of virus consists of capsid protein C with molecular weight of 14 kDa [6], which carries single stranded (+) sense RNA with length of approximately 11 kb [5]. RNA contains one open reading frame (ORF) flanked by 5´ and 3´ untranslated regions and encodes three structural proteins (E, M, C) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). NS3 is helicase and NS5 is RNA-dependent RNA polymerase and both form the polymerase complexes during the infectious cycle, which are probably associated with membranes together with proteins NS1 and NS2A. Moreover, NS3 protein together with NS2B protein provides virus-specific serine protease activity for the cleavage of the newly formed virus polyprotein and NS4A and NS4B nonstructural proteins probably supply appropriate orientation of polyprotein in the intracellular membranes [7]. NS1 nonstructural glycoprotein is usually localized inside or on the surface of the infected cell and induces the response of the immune system of the body by producing complement-fixing antibodies agains tick-borne encephalitis virus [8].

Individual subtypes of the tick-borne encephalitis virus have the same molecular structure, but differ in neurovirulence (especially Siberian and Far Eastern virus subtype when compated to the European subtype), which is caused by 3% difference in the amino acid sequence in E structural glycoprotein and also by changes in C-terminal part on the NS1 nonstructural glycoprotein [9].

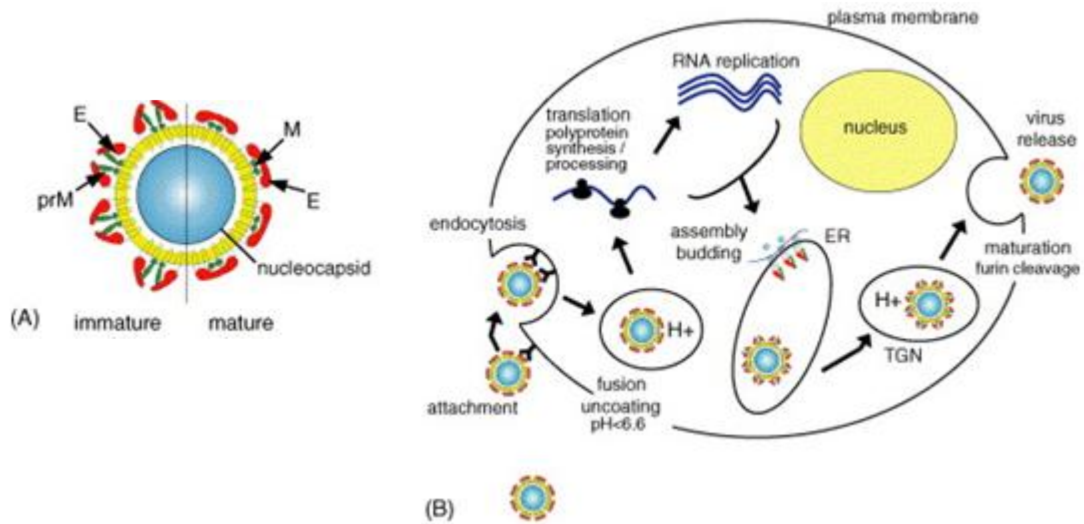

Figure 1: (A) Structure of the immature virion containing E (envelope), prM (precursor of membrane) and C (nucleocapsid or capsid) glycoprotein in comparison with structure of mature virion containing E, M (membrane) and C glycoprotein. (B) Replication of virus particle in the host cell. E protein of virion interacts with receptors on the surface of the host cell and virion is transported into the cell by endocytosis. Membrane of endosomal vesicle and E protein of virus are fused under acidic conditions, RNA is released into cytoplasm, uncoated and translated using negative-strand RNA template to form new viral RNA. Immature virion is formed in ER (endoplasmatic reticulum), transported throught cellular secretory pathway to acidic vesicles of TGN (trans-Golgi network), where the mature infectious virion is formed by furin protease cleavage. Afterwards, the mature virion is released from the cell. Figure was adapted from [10].

1.3. Interaction of flaviviruses with the host cell

Firstly, the Flavivirus E glycoprotein interacts with receptors on a surface of the host cell. Heparan sulphate (HS) receptors play probably the major role in this process. Heparan sulphate belongs to the glycosaminoglycans, which are linear, polyanionic carbohydrate chains specifically regulated on the surface of the cells of different tissues of the body. It was determined, that the flavivirus-host cell interaction is not just single receptor mediated process, because the virus can infect also cells lacking HS receptors, but the exact process of virus mediation to the host cell is still unknown [11]. The virion is then transported into the cell by clathrin-mediated endocytosis [12] and placed into endosomal vesicle. Vesicle is acidified, which changes the conformation of E glycoprotein and rearanges its dimers to trimers. Envelope and membrane of the vesicle is fused and RNA of the virion is released into cytoplasm of the host cell. Viral RNA is uncoated and replicated using negative-strand RNA templates formed from viral positive-strand RNA. Subsequently, new immature virion is formed in endoplasmatic

reticulum of the host cell and contains E, prM and C glycoproteins. Then it is transported throught cellular secretory pathway and prM protein is cleaved by furin protease in acidic vesicles of trans-Golgi network, so that M proteins are formed. Finally, the mature, infectious virion containing E, M, and C glycoproteins is released from the host cell [10] (Fig. 1(B)).

1.4. Ecology of TBEV

Tick-borne encephalitis virus belongs to the so-called Arthropod borne virus group, which means that the natural cycle of these viruses includes arthropod vectors which transport infectious virus particles into the body of vertebrate host. In the case of the European subtype, the main arthropod vector is *Ixodes ricinus* tick species and in case of the Far Eastern and Siberian subtype the *Ixodes persulcatus* tick, but also other tick species are involved in tick-borne encephalitis virus transmission. *I. ricinus* is the most dominant tick species in Europe. Natural habitat of this species are deciduous and mixed forests containing oak trees and larger mammals [13] and their main feeding peaks occur 2 times per year: firstly from May to June and then also from September to October [1]. On the other hand, *I. persulcatus* species are occuring in the Ural, Siberia and the Far East [1] and its natural habitat are mixed, coniferous and taiga forests with spruce trees [14]. These ticks prefer warm and relatively humid conditions and their main feeding period occurs from May to June [1], [3]. Generally, the northern and height border of occurence of *Ixodes* ticks is raising due to the changes in the global climax. According to the newest studies, the height border raised above 1100 meters above the sea level [15].

In general, the life cycle of tick includes 3 stages: larva, nymph and adult. Each stage is fed on blood of different vertebrate hosts and it takes approximatelly 1 year until it develops to the next life stage. Once the virus enters the tick body at some stage, it is transported also into other stages or can be transmitted also from infected female to the egg and into the vertebrate hosts, but dissease is not developped in the tick body. The natural reservoir of the tick-borne encephalitis virus are organisms, which develop viremia for the long time period, but do not become clinically ill. Human can develop disease with viremia, but they are not included in the virus natural cycle [3].

1.5. Tick-borne encephalitis

Virus can enter the human body mainly by bitting of the tick, whose saliva contains virus particles. The other possible ways of infection are inhalation the infected aerosol or consumation of cheese or unboiled milk from infected goats, sheep or cows [3]. In the case of tick bite, the virus particles firstly enter epidermal Langerhans cells, which transport them to the draining lymph nodes [16]. Subsequently, the viremia is formed following virus replication in the lymph system and by its transportation to other tissues. In the case of human, the virus is neuropathogenetic and invades the central nervous system (CNS) which is infected in the stage of high-level viremia [10]. The process of crossing the blood-brain barrier was not well described up to now, but it was determined, that at later stages of the dissease the blood-brain barrier is getting more permeable for virus particles [3]. Neurons are primary targets for the virus particles in developing tick-borne encephalitis disease. They are injured due to the cytotoxicity of the virus and due to the induction of immunopathogenetic response, sometimes ending in their chronical damage [10].

Encephalitis caused by different subtypes of tick-borne encephalitis virus differs in the later stages of the dissease. Generally, after penetration of the virus to the human body, the incubation period lasts for 7 – 14 days. Then the first phase (viremia) of the disease is developed with characteristic symptoms as influenza, raise of the body temperature, headache, vomiting, fatigue, lasting approximatelly 1 – 2 days. Subsequently, the symptom-free phase follows for the next week and can continue by the second phase of the disease, which includes various neurological symptoms [3]. In detail, tick-borne encephalitis can be divided into several forms:

- *Febrile form:* The shortest form, without neurological symptoms followed by complete recovery.
- *Meningeal form:* Similar to the febrile form, including symptoms like headache, nausea and photophobicity. This form of tick-borne encephalitis lasts for 7 – 14 days and patients are fully recovered afterwards.
- *Meningoencephalitic form:* Not very common form, but with symptoms which are difficult to treat like fibrillar contractions, stomach bleeding, bradycardia or epileptic fits. This form is fatal in 30 % of all cases, damage of CNS is typical and recovery is very slow.

- *Poliomyelitic form:* Very complicated form including many neurological symptoms. It begins with the fatigue, muscle contractions, weakness or numbness of the limbs, continues with muscle atrophy and ends with the paralysis of parts of the body. Only half of the patient shows partial recovery from the symptoms.
- *Polyradiculoneuritic form:* Biphasic form, which includes the first phase with symptoms like elevated temperature, vomiting, headache, the symptoms-free stadium and second phase with the neurological symptoms. The recovery is usually complete.
- *Chronic form:* This form was reported only in case of Far Eastern and Siberian subtype of the tick-borne encephalitis virus. Neurological symptoms of acute phase of the encephalitis disease are lasting for long time period or they are developing for many years [1].

The fatality in the case of Far Eastern subtype is 35 % and complete recovery is 25 %. In the case of Siberian subtype the fatality is 2 % and complete recovery occurs in 80 % of all cases. Fatality on the European subtype is nowadays lower than 2 %. Tick-borne encephalitis is subclinical or asymptomatic in 70 – 95 % cases [3].

1.6. Geographical distribution of TBEV

The tick-borne encephalitis is mainly appearing in various parts of Central Europe, Scandinavia and Northern Asia. The most attacked parts of Russia are Ural and Western Siberia. Some cases of TBE were reported also in Turkey and Japan. The countries without any reported case of this disease are Great Britain, Ireland, Belgium, Iceland, Netherlands, Luxemburg, Spain and Portugal [3].

1.7. Treatment and prevention

The treatment of the tick-borne encephalitis is exclusively symptomatic because there are no effective antivirotics or other treatment agents available. Due to this fact, the prevention of the disease is extremely important. Mostly effective and widely used vaccines contain inactivated virus particles or parts of the virus body, usually the envelope (E) protein. When producing such vaccine, virus particles are firstly replicated in the chick embryos, filtrated, inactivated in formaldehyde and purified by

ultracentrifugation to avoid any allergic reaction. Subsequently, the pure viral particles are stabilized by chemical substances, which differ with the producer of the vaccine. In Europe, there are 2 vaccines widely used: FSME-IMMUN (Baxter) and Encepur (Novartis) [3]. These vaccines contain inactivated European virus subtype and they showed to be very effective as TBE was almost fully eliminated in Austria, due to the extensive immunization. In Russia, there are used vaccines with inactivated Far Eastern virus subtype, for example EnceVir (Virion), but their efficiency is not as high as in Europe, probably, because of the high virulence of Far Eastern virus subtype. Moreover, there were clinical trials in Russia with live attenuated Langat virus-based vaccine. The results showed longer immunization without need of re-vaccination comparing to the vaccines using inactivated virus particles, but on the other hand, development of the encephalitic processes in the vaccinated persons was determined. Due to this, the usage of live attenuated vaccines against TBEV was discontinued [1].

The future trends in development of vaccines and antivirotic agents against TBEV includes using of naked plasmid DNA or nonstructural NS1, NS3, NS5 proteins which induces immune response of T-cells [1].

1.8. Principles of methods used for testing of TBEV in the experimental work

1.8.1. CellTiter-Glo® Luminescent Cell Viability Assay (HTS assay)

The CellTiter-Glo® Luminescent Cell Viability Assay or High Throughput Screening assay is method for determining the metabolically active cells by quantification of ATP molecules produced by cells. The amount of ATP produced is proportional to the number of viable cells. This method is based on luciferase reaction with ATP which generates „glow-type“ luminescent signal which can be measured by luminometer. The luciferase reaction is caused by the CellTiter-Glo® reagent which induces lysis of the cell membranes, inhibition of endogenous ATPases which would destroy any ATP molecule and provides luciferin, luciferase and other reagents due to which bioluminescent reaction can take place [17].

1.8.2. CellTiter 96® AQueous One Solution Cell Proliferation Assay (Cytotoxicity assay)

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is a colorimetric method widely used for determination of amount of viable cells in the cytotoxicity assays. The CellTiter 96® AQueous One Solution contains MTS tetrazolium compound (Owen´s reagent) (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) stabilized by electron coupling reagent PES (phenazine ethosulfate). NADH or NADPH produced by dehydrogenases in metabolically active cells reduces MTS to formazan which is soluble in tissue culture medium. Due to formazan production, which amount is proportional to the number of living cells, the absorbance at 490 nm wavelength can be measured and hence the amount of living cells can be determined [18].

1.8.3. Plaque assay

Plaque assay is technique for determining the concentration of viral particles (viral titer) in the solution. 10-fold dilutions of viral stock solution are applied to the cell monolayers, which are subsequently covered with nutrient solution. After incubation, the cell release newly formed viral particles, which infect neighbouring cells. Generally, each infectious particle forms circle of the infected cells, which is called plaque. This method is useful only for viruses which cause visible cell damage, so that living cells can be stained and viral titer in PFU (plaque-forming unit) per mililiter can be determined by counting of the number of plaques [19].

2. AIMS OF THESIS

- Study of inhibition effect of chemical substances on tick-borne encephalitis virus using HTS assay.
- Determination of cytotoxicity of effective chemicals on PS cells.
- Determination of viral titer using plaque assay.

3. MATERIALS AND METHODS

3.1. Cell cultures

Pig kidney epithelial (PS) cell culture [20] was cultivated in L15 medium (Leibowitz) (Sigma-Aldrich) with 3 % of fetal calf serum (FCS), 1 % of antibiotics (penicilin and streptomycin) (Sigma-Aldrich) and 1 % of glutamine at 37 °C.

3.2. Virus strain

Tick-borne encephalitis virus, HYPR strain, was used for testing of the effect of chemical substances. HYPR belongs to the Central European subtype which was firstly isolated in Czechoslovakia in 1953 from the blood of 10-year-old boy infected by this virus strain. It is highly passaged in laboratories and it shows high virulence comparing to the Neudoerfl strain, which also belongs to the Central European subtype [21].

3.3. Chemical substances tested

All chemical substances were synthetized in the Institute of Chemical Technology in Prague and all of them were provided in solid state, except substance MK 216 which was provided in liquid state. Their structures can be seen in Fig. 2A and 2B.

Substances were weighed and dissolved in 99.9 % DMSO, so that 25 mM concentration was obtained. Some of them had to be dissolved by heating in water bath. All chemicals were subsequently diluted with L15 medium + 3 % FCS + 1 % ATB + 1 % glutamine to the final concentration 10, 20 and 50 μM.

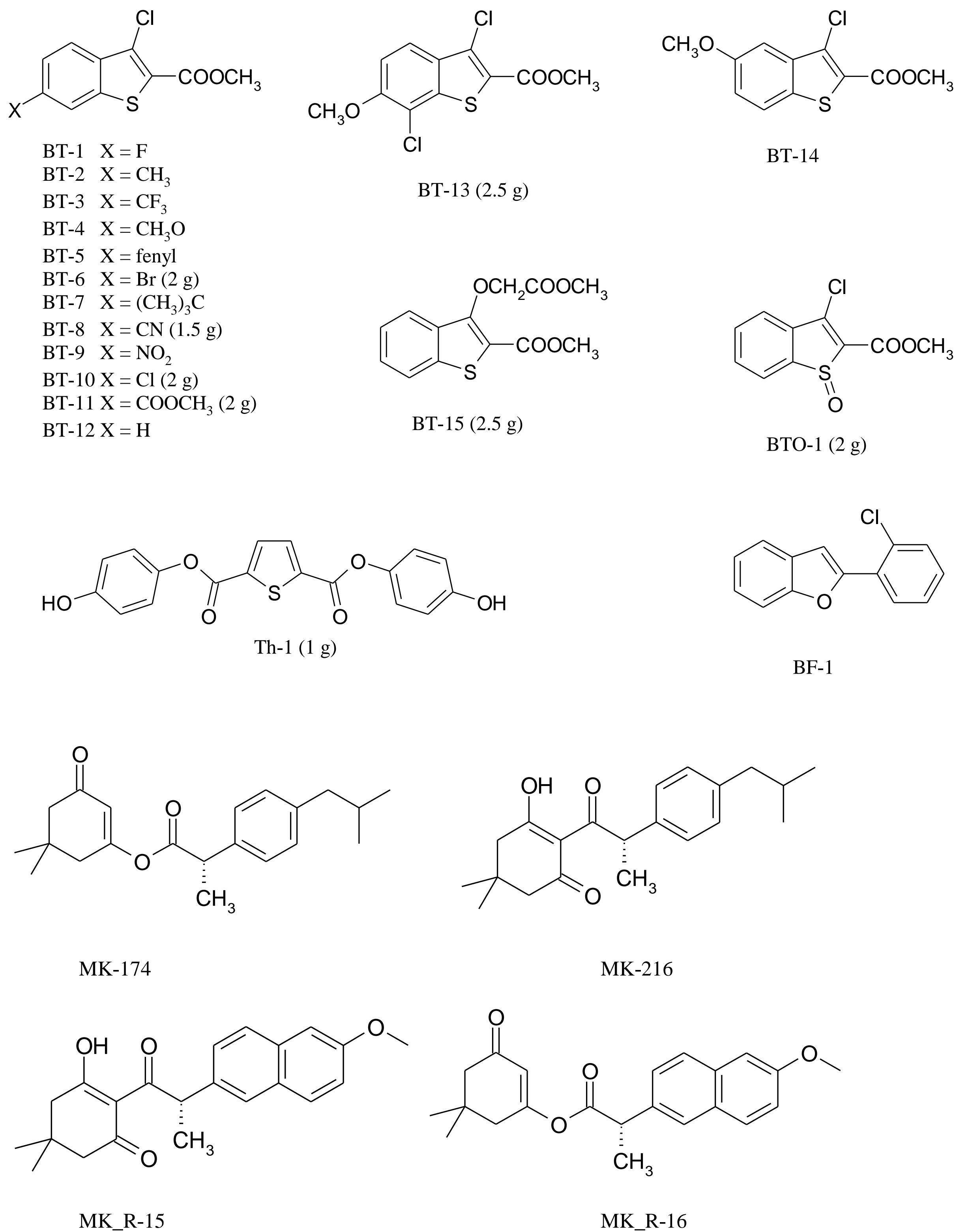

Figure 2A: Structure of chemical compounds BT-1 - BT-12 differing always in one functional group and structure of molecules BT-13, BT-14, BT-15, BTO-1, Th-1, BF-1, MK-174, MK-216, MK_R-15 and MK_R-16.

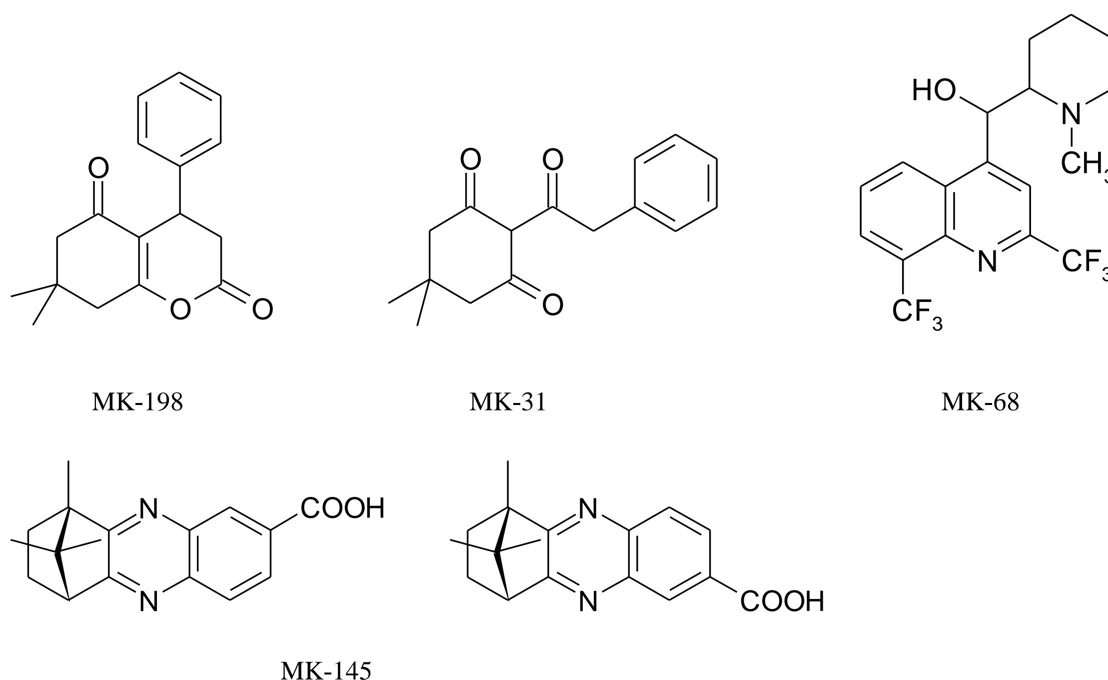

Figure 2B: Structure of substances MK-198, MK-31, MK-68 and MK-145 which was provided as a mixture of isomeres.

3.4. CellTiter-Glo® Luminescent Cell Viability Assay (HTS assay)

Firstly, the appropriate amount of cells per well was detected by addition of 100 µL of cell culture containing $1E^{+3}$, $1.25E^{+3}$, $2.5E^{+3}$, $5E^{+3}$, $7.5E^{+3}$, $1E^{+4}$, $1.25E^{+4}$ and $2.5E^{+4}$ cells respectively into 96-well plate. Cells were incubated for 24 hours at 37 °C under 0.5 % CO_2 atmosphere, plates were then equilibrated to room temperature and 100 µL of CellTiter-Glo® reagent (Promega) were added into each well. Plates were shaken by orbital movement, incubated for 10 minutes at room temperature and luminescence was recorded. Number of cells in the beggining of linear region was used for further experiment.

HTS assay was performed by adding 100 µL containing $1.25E^{+4}$ cells into the wells of 96-well plate. Cells were incubated for 24 hous at 37 °C under 0.5 % CO_2 atmosphere. Afterwards, medium was taken out of wells and 50 µL of chemical substances were added so that the final concentrations of each substance were 5, 10 and 25 µM respectively. In control wells the medium was changed for 100 µL of the fresh one. Subsequently, 50 µL of virus with multiplicity of infection (MOI) approximatelly 8 were added, plates were incubated at the same conditions for 3 days, then equilibrated to room

temperature, mixed with 100 μL of CellTiter-Glo® reagent, incubated for 10 minutes at room temperature and luminescence was measured using Microplate Luminometer Orion II, Bethold Detection Systems.

3.5. CellTiter 96® AQueous One Solution Cell Proliferation Assay (Cytotoxicity assay)

Like in the case of HTS assay, the appropriate amount of cells per well was determined at first. 100 μL of cell culture containing $4E^{+3}$, $5E^{+3}$, $1E^{+4}$, $2E^{+4}$, $3E^{+4}$ and $4E^{+4}$ of cells respectivelly, were given into the wells of 96-well plate. Cells were incubated for 24 hour at 37 °C under 0.5 % CO_2 atmosphere. Afterwards, 20 μL of the CellTiter 96® AQueous One Solution (Promega) were added into each well, plate was incubated for 1 hour at the same conditions and absorbance at 490 nm was determined. After processing data by linear regression, value of $1E^{+4}$ cells per well was chosen for cytotoxicity assay because it appeared at the lower end of linear range.

Only substances which showed the inhibition effect in the HTS assay were tested for their cytotoxicity effect on PS cells. These substances were BT-8 at 25 μM concentration, BF-1 at 5μM, MK-174 at 25 μM, MK-145 at 5, 10 and 25 μM concentration. The range of tested concentrations of substances was widened and substances were diluted with medium to additional concentrations around their effective concentration. Cytotoxicity of DMSO on cells was tested using medium containing 0.24, 0.14 and 0.016 % of DMSO. Moreover, the wells containing only cells with medium were used as positive control. The procedure was following: each well of 96-well plate was seeded with $1E^{+4}$ cells, which were subsequently incubated for 24 hours at 37°C under 0.5 % CO_2 atmosphere. Afterwards, 50 μL of medium were removed and 50 μL of substances or medium containing DMSO were added. 50 μL of pure medium were added into the controll wells. Plate was incubated for 48 hours at the same conditions. Afterwards, 20 μL of the CellTiter 96® AQueous One Solution were given into each well, plate was incubated for 1 hour at the same conditions and absorbance at 490 nm was determined using ELISA reader Infinite M200, TECAN.

3.6. Viral titer reduction assay

Only substances which showed inhibition effect on virus and no cytotoxicity effect on PS cells were used further in viral titer reduction assay. These substances were BT-8 and MK-174 at 20, 22.5, 25, 27.5 and 30 μM concentration and MK-145 at 8, 10, 15, 18, 20, 22.5, 25, 27.5 and 30 μM concentration.

200 μL of cell culture containing $2E^{+4}$ cells were given into each well of 96-well plate and incubated for 24 hours at 37 °C under 0.5 % CO_2 atmosphere. Afterwards, 150 μL of medium were taken out, 50 μL of virus with MOI 0.1 and 100 μL of substances were added into each well except the controll wells. Positive control consisted of cells with medium and negative control contained cells with virus. Two identical plates were formed and one was incubated for 2 days and the second one for 3 days at 37 °C under 0.5 % CO_2 atmosphere. After incubation, supernatants were transferred to eppendorf tubes, frozen at -72 °C and processed in plaque assay.

3.7. Plaque assay

Each supernatant from viral titer reduction assay was diluted 100x (10 μL of supernatant + 990 μL of L15 medium). 180 μL of L15 medium + 3 % FCS + 1 % ATB + 1 % glutamine were added into each well of 24-well plate. Then ten fold dilutions of diluted viral supernatant from viral titer reduction assay were performed always in 6 wells (20 μL of viral supernatant mixed with 180 μL of L15 medium in well, then 20 μL of this mixture were mixed with medium in the other well, etc). Subsequently, 300 μL of cell culture containing $1.2E^{+5}$ cells were added into each well, plates were incubated 4-5 hours at 37 °C under 0.5 % CO_2 atmosphere and afterwards 400 μL of nutrient substance (CMC:L15 medium in ratio 1:1) were added dropwisely into each well. Plates were incubated 5 days at the same conditions as before. After incubation, plates were washed with saline (8.5 – 9 g of NaCl dissolved in 1 L of redistilled water) and cell monolayers were stained with naphtalene black solution. Subsequently, plaques were counted and viral titer for each chemical substance tested was determined by formula:

*Viral titer = (amount of plaques*dilution*1000)/180 mL [pfu/mL]*

3.8. Statistical analysis

All results were analyzed by program Statistica 10, StatSoft, using independent two sample *t*-test. Values significant at the level $p = 0.05$ are marked as the red column in the graphs.

4. RESULTS

4.1. HTS assay

High Throughput Screening assay was performed according to the procedure described in section 3.4. Each chemical substance was measured three times, average luminescence was calculated and results of effective substances were given into graph (Figure 3) as well as value for negative control (cells + virus) for visual comparison of the inhibition effect.

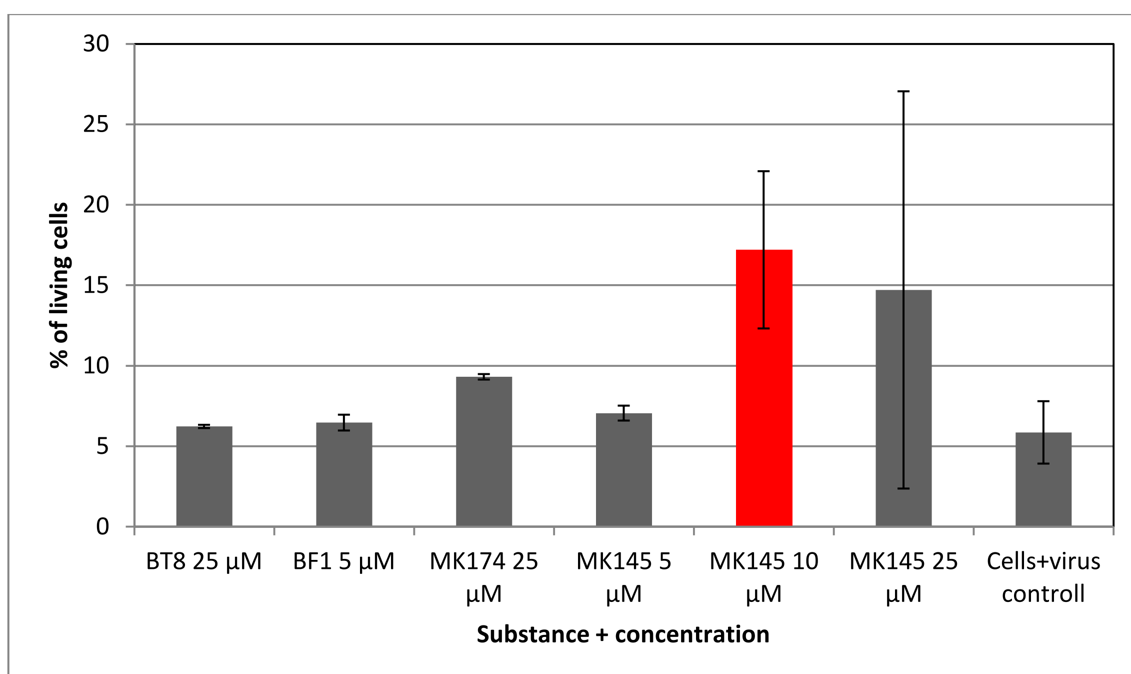

Figure 3: Graph showing effect of chemical substances (only effective ones)

According to the results of HTS assay (Figure 3), cells treated with six substances showed higher luminescence than the negative control (cells incubated with virus), namely BT8 (25 µM), BF1 (5 µM), MK174 (25 µM), MK145 (5, 10 and 25 µM). Even though, only MK145 with 10 µM concentration showed significant inhibition effect at the level $p = 0.05$. It is important to say, that measured luminesce of living cells was in ranges of $1E^{+6} - 1E^{+7}$ which resulted in high values of standart deviation (MK145 - 25 µM). Nevertheless, all named substances were tested further by Cytotoxicity assay.

4.2. Cytotoxicity assay

Substances which showed higher luminescence comparing to the luminescence of negative control in HTS assay were tested further by cytotoxicity assay, according to the procedure described in section 3.5. Compounds were diluted to two additional concentrations around the effective one. For example, BF1 was diluted to 2 and 8 μM because 5 μM was the effective concentration. Viability of cells after application of substances compared to DMSO control can be seen in Figure 4.

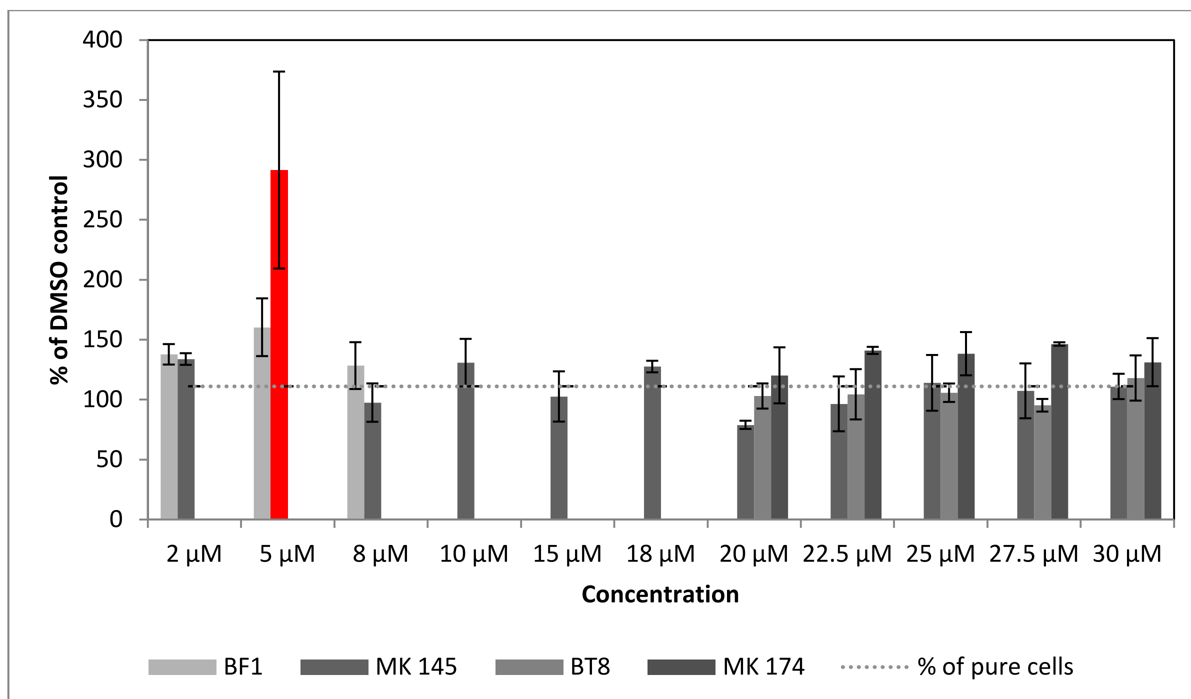

Figure 4: Amount of living cells (%) after application of chemical substances comparing to the DMSO control and positive control (cells + medium).

When comparing viability of PS cells after their treatment with chemical substances with the positive control (cell culture) (Figure 4), we can see that most of the substances are not toxic for PS cells. On the other hand, there can be few exceptions found. First of them is viability of cells treated with MK145 with 5 μM concentration which was rapidly increased to almost triple amount comparing to the viability of pure cells. This value is also significantly different ($p = 0.05$) from the viability of cell culture. This deviation was probably caused by the toxicity of substance which activated protection mechanism of cells and led to the enhanced enzymatic productivity in mitochondrias [22]. Similar situation occurred also in the case of BF1 substance. Althouh these viability values did not show significant difference from cell culture, they were not tested further by plaque assay. Oppositely, viability of cells treated with MK145 (20 μM) was decreased. This

substance was still processed in the plaque assay, where it showed the fatal toxicity to the cells (Figure 5 and 6). MK145 (all concentrations above 8 μM), BT8 and MK174 (all concentration tested by cytotoxicity assay) were tested further by Plaque assay.

4.3. Plaque assay

Substances which showed no or little toxicity to cells in cytotoxicity assay were processed by viral titer reduction assay as desribed in section 3.6. Reduced viral titers were then examined by Plaque assay using the procedure described in section 3.7.

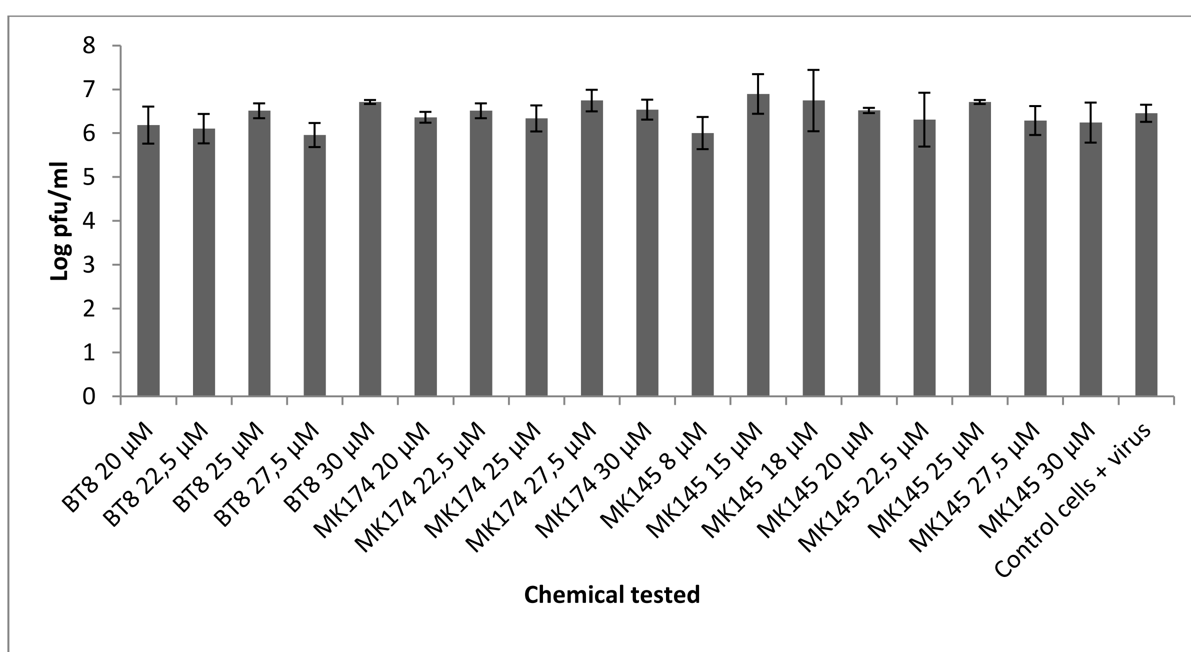

Figure 5: Viral titers (log pfu/mL) of supernatants from viral titer reduction assay incubated for 2 days.

Substances incubated for two days with viral and cell culture showed no significant decrease ($p = 0.05$) in viral titer comparing to the negative control (Figure 5). MK145 (10 μM) was also tested, but no cells were grown in the first wells of 24-well plate, so plaques could not be identified. This problem could be caused by cytotoxicity of MK145 (10 μM), but as it did not show high deviation from the positive controll in the cytotoxicity assay, the inhibition of cell growth had to be caused by contamination of supernatant from viral titer reduction assay.

According to the Figure 5, the lowest viral titer occurred in supernatant from cell culture treated with MK145 (8 μM) and BT8 (27.5 μM). On the other hand, MK145 (15 and 18 μM) even higher virus titer.

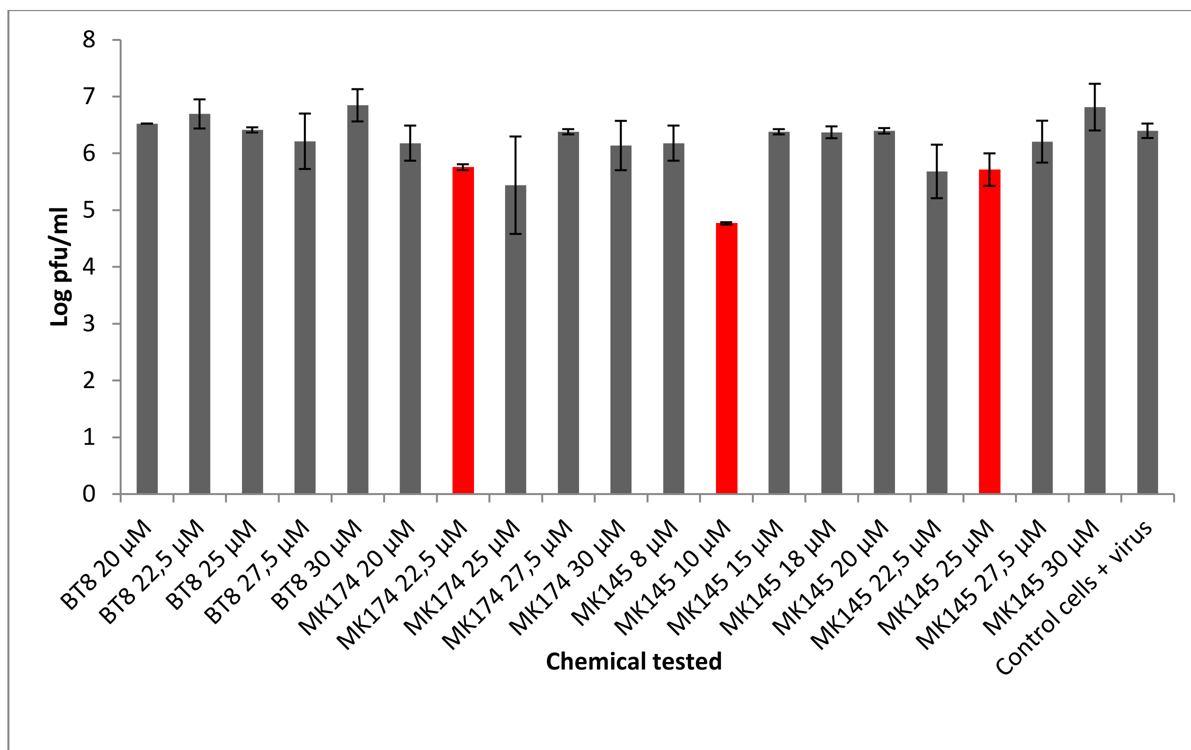

Figure 6: Viral titers (log pfu/mL) of supernatants from viral titer reduction assay incubated for 3 days.

Substances incubated for 3 days in the viral titer reduction assay resulted in three cases with significant inhibition effect ($p = 0.05$) on HYPR strain of the tick-borne encephalitis virus (marked as red columns in the Figure 6). The most significant decrease of viral titer was observed in supernatant from culture treated with MK145 (10 µM). Another effective substances were MK174 (22.5 µM) and MK145 (25 µM). MK174 (25 µM) showed even lower viral titer, but the standart deviation was quite high, so the values are not as reliable as those of significant concentrations. Generally, substances which showed lower number of viable cells in cytotoxicity assay comparing to the pure cell culture, enhanced formation of virions in viral titer reduction assay which resulted in formation of multiple plaques in plaque assay. On the other hand, substances causing enhanced viability of cells (but not as high as values of viability of cells treated with BF1) showed inhibition effect on tick-borne encephalitic virus in viral titer assay. The reason for that can be either little toxicity of substance which cause enhanced enzymatic activity of cell which has prophylactic effect for cell or protective ability of the substance.

5. DISCUSSION

Testing of antivirals against tick-borne encephalitis virus do not reveal many chemical substances with antiviral effect. Flaviviruses tested frequently include the most pathogenic viruses among this virus genus like Dengue virus, West Nile virus or Yellow Fever virus.

In vitro studies of the tick-borne encephalitis virus infection usually work with pig kidney epithelial (PS) cells [23], [24]. On the other hand, other Flaviviruses are tested either on Vero cells (kidney epithelial cells isolated from African green monkey) [25], [26] or on baby hamster kidney fibroblasts (BHK-21) [27], [28]. Testing methods used in this bachelor thesis are mostly identical with the methods generally used for testing of inhibition effect of chemical substances against Flaviviruses. These techniques include High Throughput Screening assay, Cytotoxicity (Viability) assay and Plaque assay. CellTiter-Glo reagents (Promega) which were applied are used for testing of various types of viruses like Influenza virus [29] or Bluetongue virus [30], but can be effective also when studying Dengue virus [31]. Although that usage of these kits is very easy and fast, Flaviviruses are mainly tested with different reagents [26], [24], [27]. In the case of Plaque assay, approximatelly the same reagents and procedures are employed.

Effective antivirals against Flaviviruses are mainly complex organic substances or bases of DNA or RNA which can be inserted into the viral genetic information and subsequently degrade the virus. Such case was reported in [24] where artificial RNAses which exhibit ribonuclease activity *in vitro* degraded the genome of the tick-borne encephalitis virus (strain Sofjin) after penetrating the viral membrane. The strongest inhibition effect had RNAse Dtr12 which contains two cyclohexane rings with two nitrogen heteroatoms. MK145 tested in this thesis which was effective on HYPR strain of tick-borne encephalitis virus also contained cyclic ring consisting of two nitrogen heteroatoms and four carbons which was bind to one benzene ring and one bicyclic ring. Another effective chemical containing nitrogen atom built-in cyclic ring was reported in [32] where imino sugar derivate inhibited replication of Dengue virus. Interferon INF-α which is currently used for treatment of chronic Hepatitis C showed inhibition effect against Dengue virus, Yellow Fever virus, Japanese encephalitis and tick-borne encephalitis virus [26]. Another from the variety of tested compounds was NITD-982 which inhibits host dihydroorotate dehydrogenase artificial for biosynthesis of pyrimidine. This ability resulted in the antiviral activity of the

substance against Dengue virus and moreover against alphavirus, rhabodovirus and HIV. Triaryl pyrazoline, compound containing five membered ring with two nitrogen heteroatoms, inhibited replication of West Nile virus RNA in first 10 hours post infection. Significant inhibition effect of this compound rapidly decreased when treating the cell culture after 10 hours p. i. Similarly, the West Nile and Kunjin virus were significantly inhibited by secondary sulfonamides [28]. To conclude, the most effective antivirals against Flaviviruses are organic molecules comprising cyclic ring with at least one nitrogen heteroatom.

Even when the results of *in vitro* experiments show inhibition effect of the substance, *in vivo* experiments are always challenging. This is the case of study of substance NITD-982 which revealed inhibition effect against Dengue virus *in vitro*, but showed no inhibition effect when tested in DENV-AG129 mice [31]. This is the standard situation because living organism is complex system exhibiting many processes which can not be simulated in *in vitro* experiments. On the other hand, *in vivo* research reveals much important information on how the infection is transported within the body. Research of interaction of tick-borne encephalitis virus antibodies with macrophages infected by this virus in BALB/c female mice revealed that macrophages are not responsible for carrying infection directly into the central nervous system, but also early extracellular step has to be involved [23].

6. CONCLUSION

Twenty-six chemical substances were tested for their inhibition effect against HYPR strain of the tick-borne encephalitis virus. Cells treated with six of them showed higher viability values than the negative control (cells infected with virus) in the High Throughput Screening assay. Furthermore, cytotoxicity of these chemicals on PS cells was determined by Cytotoxicity assay and only three substances with different concentrations were processed further in Plaque assay. The results of this assay revealed two substances with three different concentrations with significant inhibition effect against the virus. These substances were MK174 with 22.5 μM concentration and MK145 with 10 μM and 25 μM concentrations in DMSO. MK174 is an ester containing one aromatic ring (Figure 2A) and MK145 is carboxylic acid containing three rings: bicyclic, aromatic ring and aromatic ring with two nitrogen heteroatoms (Figure 2B). MK145 was provided as a mixture of isomeres which could be the cause of its restraint effect against TBEV. Several other substances were also effective, but their standart deviation was too high which makes these results not very reliable.

Substances MK174 and MK145 showed statistically significant inhibition effect ($p = 0.05$ at some concentrations in the culture) against tick-borne encephalitis virus. To determine whether they can serve as antivirals against this virus, they have to be tested further by *in vivo* experiments and other clinical trials have to be done.

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