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The effect of type-I interferons, and the antiviral drugs ribavirin and favipiravir on rabies virus replication in mouse neuroblastoma (N2A) cells

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1 Abbreviations

BSL: Biological Safety Level
CNS: Central Nervous System
DMEM: Dulbecco's Modified Eagle's Medium
FCS: Foetal serum
G protein: Glycoprotein
IFNAR: IFN-α receptors
IFN: Interferon
IL: Interleukin
IMPDH: Inosine monophosphate dehydrogenase
IPS-1: IFN-β promoter stimulator-1
IRF-3: IFN Regulatory Factor-3
ISG: Interferon Stimulated Genes
ISGF3: Interferon stimulated gene factor 3
ISRE: IFN stimulated response element
IκB: Inhibitor of NFκB
JAK-1: Janus kinase 1
L protein: Large protein (RNA-dependent RNA polymerase)
MAVS: Mitochondrial Antiviral Signalling Molecule
M protein: Matrix protein
MOI: Multiplicity of infection
N Protein: Nucleoprotein
NFκB: Nuclear Factor Kappa B
P protein: Phosphoprotein
PRR: pattern recognition receptors
RABV: Rabies Virus
RdRP: RNA dependent RNA Polymerase
RLR: Retinoic Inducible Gene-I like receptors
RLR: RIG-I like receptors
RMP: Ribavirin monophosphate
RTP: Ribavirin triphosphate
STAT: Signal Transducer and Activator of Transcription
Th1: T-helper 1 cell
Th2: T-helper 2 cell
TLR: Toll-like receptors
TNF-α: Tumour necrosis factor- α
TYK-2: Tyrosine kinase 2

2 Introduction

Rabies is a viral zoonotic disease that has been known to man for about 4000 years. Still today, rabies remains a feared disease that poses a serious threat both to human and veterinary public health, especially in developing countries. It is responsible for more than 55 000 human deaths around the world every year; the most among all zoonoses. In endemic countries, 40-50% of the human deaths caused by rabies are children below the age of 15 years (Fooks et al., 2014).

There is no effective treatment regimen, and the disease has an almost invariably fatal outcome after the onset of clinical symptoms. To date, only 10 patients who had developed clinical rabies has survived the disease (see **3.1.3**). Nine of these patients received either pre- or post-exposure vaccines. One patient who survived did not receive pre- nor post-exposure prophylaxis, but efforts to establish a reliable therapeutic procedure based on the "Milwaukee protocol" used in this case failed after several unsuccessful attempts. (Jackson, 2013; Fooks et al., 2014).

Type I interferon (IFN-α and IFN-β) -production by cells of the body is a known direct response to viral infections. The production of IFN-α and IFN-β stimulates a signalling pathway, leading to the up-regulation of expression of hundreds of interferon stimulated genes (ISG), which exert their antiviral effects by means of various mechanisms (Randall and Goodbourn, 2008).

Previous experiments have shown that rabies virus (RABV) infection triggers a type I interferon response in neuronal cells in the early stages of infection (Chopy et. al., 2011). However, this effect is transient, and diminishes as the infection progresses, and the influx of the host's immune cells is not enough to control the progress. This leads to the hypothesis that the virus has unique mechanisms by which it can evade the host interferon response. This hypothesis has been investigated, and it is suggested that that viral proteins, especially the P protein, are responsible for this evasion (Ito et al., 2010). Even though RABV has developed mechanisms to avoid the host immune response, the transient interferon response that has been detected in infected cells, leads us to believe that the mechanisms are not perfect, and opens the possibility of using interferons in potential treatment of RABV infection.

Ribavirin is an antiviral drug with mutagenic activity as its suggested main mechanism of action. It is a guanosine analogue; a competitive inhibitor of viral RNA polymerase, with a broad-spectrum antiviral activity. Considering that RABV is capable of avoiding the host immune system and infection induces minimal inflammatory response in the nervous tissue, there is a possibility that modulating the inflammatory response of the host can contribute to the clearance of viruses from the central nervous system (CNS). Ribavirin has been demonstrated to have immunomodulatory effects (Te et al., 2007). In addition, ribavirin stimulates the expression of ISGs with antiviral properties (Appolinário et al., 2013).
Ribavirin is proved to be effective in treatment of several viral infections. Experiments on ribavirin treatment of chronic hepatitis C in combination with interferons have shown an enhanced activity (Te et al., 2007). The underlying mechanism of this effect is unknown, but it raises the question if this enhanced effect can occur in the treatment of other viral infections; like rabies.

Favipiravir, or T-705, is another drug with known antiviral effect against a wide range of RNA viruses. Like ribavirin, favipiravir is also suggested to be purine analogue that targets and inhibits RNA dependent RNA polymerase (RdRP). Viruses that are resistant to other antiviral drugs like oseltamivir and amantadine have been shown to be susceptible to favipiravir (Baranovich et al. 2013). These factors make favipiravir an antiviral drug considered promising against many RNA viral diseases for which there are no approved therapies.

The aim of this study was to determine the *in vitro* antiviral effect of exogenous type I interferon treatment and that of ribavirin and favipiravir on rabies-infected mouse neuroblastoma (N2A) cells. In addition to testing these compounds individually (at four different concentrations) against RABV a combination approach has been implemented using the same drugs. The goal of combining these compounds was to possibly enhance their activity in interfering with viral multiplication, as treating rabies-infected patients with interferons or ribavirin alone showed hardly any success in the past (Warrell et al., 1989).

3 Literature review

3.1 Rabies

3.1.1 The rabies virus

The rabies virus (RABV) is a negative single stranded RNA virus, belonging to the *Rabdoviridae* family, and the *Lyssavirus* genus. The virus is neurotropic and causes fatal progressive encephalomyelitis (Niu et al. 2013). The virus is enveloped with low resistance and direct transmission is required (Fooks et al., 2014).

The genome of RABV consists of 11 928 nucleotides and it encodes five viral proteins: the nucleoprotein (N), matrix protein (M), glycoprotein (G), a large protein (L) also termed RNA-dependent RNA polymerase and a phosphoprotein (P) (Niu et al., 2013). The N, L and P proteins encapsidate the genomic RNA and form the ribonucleoprotein complex. The complex is surrounded by a lipid envelope which is associated with the M and G proteins. The N protein is responsible for protection of the RNA from recognition by the host innate immune system. The function of G protein is the binding of virus particle to the host cells at various receptors. The M protein has an important role in regulation of gene expression, and it also contributes to the facilitation of virus budding from the host cell (Albertini et al., 2011). The virus enters via an endocytic pathway, and virus transcription and replication take place in cytoplasmic inclusion bodies called Negri bodies (Lahaye et al., 2009).

3.1.2 Pathogenesis of rabies

The main route of shedding is via the saliva, and the main route of infection is through bite wounds; giving the virus direct entry to the muscle tissue (Dietzschold et al., 2008). RABV is neurotropic, it infects nearly exclusively neurons, and the neuro-invasiveness is the major characteristic of classical RABV infection (Dietzschold et al., 2005).
From the muscular tissue, the virus enters the peripheral nervous system at the neuro-muscular junctions by binding to nicotinic acetylcholine receptors. From there, the virus travels towards the central nervous system (CNS) and especially to the brain by fast

retrograde axonal transport (Tsiang et al., 1991). Many neuronal cell types are infected in a widespread distribution in the CNS. From the brain, there is centrifugal dissemination of the virus along neuronal pathways, especially the parasympathetic nerves, to different sites in the body (Jackson, 2013; **Fig. 1**). Regarding transmission, the most important is the involvement of salivary glands, from where the virus can be spread to other animals or human beings through bites. It is confirmed in literature that in exceptional cases RABV can be transmitted through milk consumption, infected urine-containing aerosols, and organ transplantation between humans (Baer, 2012).

Figure 1: Pathogenesis of rabies (Fooks et al., 2014)

1) The virus enters the muscle tissue through a bite wound 2) the virus reaches the peripheral nervous system by binding to nicotinic acetyl-choline receptors 3) the virus travels from the peripheral nervous system to the spinal cord and the brain by retrograde axonal transport 4) in the brain there is extensive replication leading to neuronal dysfunction 5a) the virus spreads via the parasympathetic nerves to the salivary glands and is shed in the saliva 5b) spread to the skin and Purkinje cells 5c) and various tissues in the body

Fatal encephalomyelitis is not necessarily accompanied by substantial inflammation. Experimental studies have showed evidence for upregulation of chemokines, cytokines and interferons in the central nervous system as a response to RABV infection, which in turn leads to an influx of immune cells to the central nervous system. However, this influx is not able to control the infection (Fooks et al., 2014).

The neuropathological changes in the central nervous system consist of mild inflammation and little neuronal degeneration. Apoptosis is a self defence mechanism in the host to limit the spread of the virus, but this does not seem to play an important role in rabies pathogenesis (Jackson, 2010). This supports the present understanding that neuronal dysfunction has a more important role in the pathogenesis than neuronal degeneration. Apart from apoptosis, another form of cell death, pyroptosis is relevant in the cellular pathogenesis of rabies, which is an obligatory proinflammatory pathway and is associated with Caspase-1 induction (Dietzschold et al, 2008; Rabuffetti et al., 2000).

Extensive virus replication in the nervous system leads to multi-organ failure and other systemic complications, but the actual mechanism of how the RABV infection causes death in the end is not well understood. Evidence for immune response is not visible until the virus has reached the CNS, and the mechanisms of how the virus avoids the immune surveillance in the periphery remains to be elucidated (Fooks et al., 2014).

3.1.3 Treatment and management of rabies

As of today, there is no effective treatment once the clinical signs have appeared. Only ten people have survived rabies, including one who had received pre-exposure prophylaxis and eight who received post-exposure prophylaxis prior to the onset of clinical signs of rabies. Two of these died within a few years. One person who did not receive pre- or post-exposure prophylaxis before the onset of clinical rabies has survived, but in that case the disease was caused by a possibly less virulent virus strain from a bat origin (Jackson, 2013; Fooks et al., 2014). The treatment included induction of coma with intravenous midazolam (Jackson, 2010), and therapy with ketamine, ribavirin, amantadine and phenobarbital (Willoughby et al., 2005). It is unknown if one or more specific therapeutic agent used played an important role in the survival of the patient. The approach has been named the Milwaukee protocol. At least 26 consequent attempts of repeating the treatment regime have failed and the outcomes have been fatal (Jackson, 2009), suggesting that the protocol is ineffective, and induction of coma should not be a routine therapy in the management of rabies (Fooks et al., 2014).

3.2 Interferons and viruses

"The interferon system is an extremely powerful antiviral response that is capable of controlling most virus infections in the absence of adaptive immunity". (Randall and Goodbourn, 2008, p. 1)

Interferons are grouped into three categories called type I, II and III. The type I interferons are a group of molecules, where IFN-α and IFN-β genes are induced directly as a response to virus infections (Randall and Goodbourn, 2008).

Type I interferons were first thought to be a factor that made cells resistant to virus infections. It is now known that cells produce type I interferons in response to viral infections, which in turn leads to an upregulation of the expression of several antiviral proteins and immunoactive cytokines. Not only do type I interferons have antiviral function; they also have a role in the activation of the adaptive immune system following a viral infection (Faul et al., 2010).

3.2.1 Induction of interferon response

A cell can recognize the presence of a virus in several different ways, and signal the induction of the interferon response. Cells that produce type I interferons have so called pattern recognition receptors (PRR), Toll-like receptors (TLR) and Retinoic Inducible Gene-I (RIG-I) like receptors (RLR) that can detect molecules associated with an invading micro-organism (Randall and Goodbourn, 2008; Takeuchi and Akira, 2008). These molecules include viral nucleic acids. The PRRs are stimulated by binding to the appropriate molecule, and activate intracellular signalling cascades that lead to transcription of type I interferons. The secreted IFN-α/β bind to receptors on neighbouring non-infected- (**fig. 2**) and infected cells, and activate a cascade that leads to upregulation of several so called IFN-α/β-stimulated genes (ISG). When the virus replicates and spread from the original infected cells to neighbouring cell that are in an antiviral state, the replication is inefficient (Randall and Goodbourn, 2008).

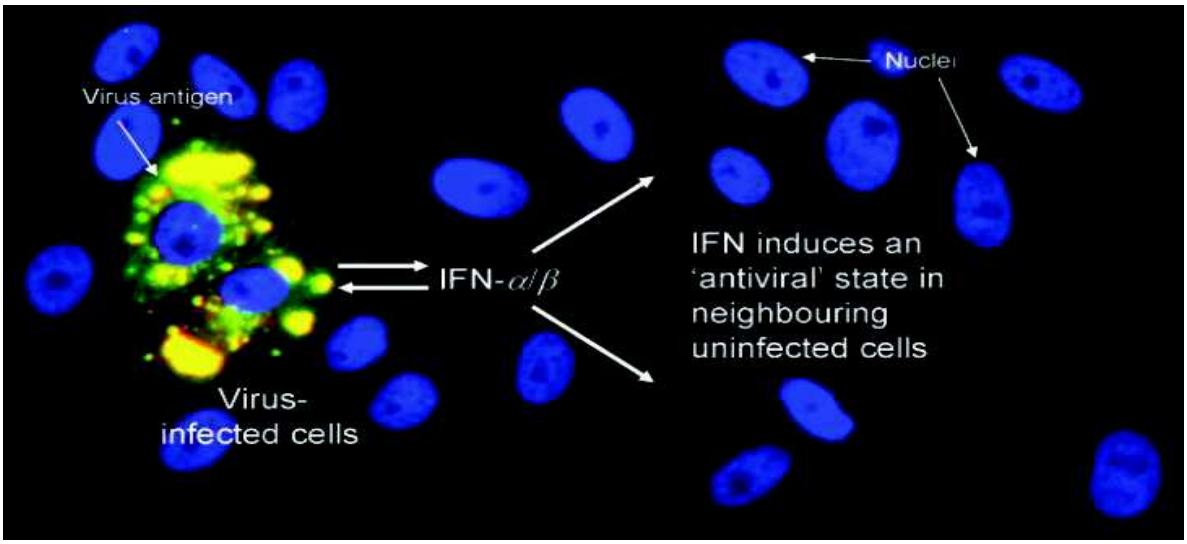

Figure 2: An overview of the type I interferon system (Randall and Goodbourn, 2008)

3.2.2 Rabies virus induces interferon response

Studies have demonstrated that double-stranded RNA is an extremely efficient inducer of interferons (Marcus and Sekellick, 1977). Rabies virus contains a negative stranded RNA, and so does not produce double stranded RNA molecules as in their normal replication cycle. However, it is likely that abnormal replication products produced by errors made by RdRP leads to some level of double stranded RNA in infected cells (Faul et al., 2010).

Another pathway by which RNA viruses can be recognized is RIG-I in the cytoplasm.
RABV is detected by RIG-I. This leads to the activation and interaction with the mitochondrial molecule IFN-β promoter stimulator-1 (IPS-1), also called mitochondrial antiviral signalling molecule (MAVS) (Haller et al., 2006). Through phosphorylation of IFN Regulatory Factor-3 (IRF-3) and activation of Nuclear Factor Kappa B (NFκB) IPS-1 induces two main signalling pathways (Chopy et. al., 2011). Before induction, IRF-3 and NFκB are both cytoplasmic. When IRF-3 is phosphorylated it undergoes a conformational change that unveils a nuclear localization signal, and is translocated into the nucleus, where it is retained until it is dephosphorylated. NFκB is held in the cytoplasm because of an inhibitor molecule called inhibitor of NFκB (IκB). Viral infection signals phosphorylation of IκB, which leads to freeing of NFκB, and NFκB is translocated to the nucleus (Randall and Goodbourn, 2008). NFκB signals the expression of inflammatory cytokines, while IRF-3 is responsible for the expression of type I interferons (Chopy et. al., 2011).

Type I interferons then bind to the IFN-α/β receptors (IFNAR). This triggers recruitment of the enzymes Janus kinase 1 (JAK-1) and Tyrosine kinase 2 (TYK-2) to IFNAR. JAK-1 and TYK-2 phosphorylate the Signal Transducer and Activator of Transcription-1 and -2 (STAT), leading to the formation of STAT-1-STAT-2 complexes (Randall and Goodbourn, 2008). These complexes are translocated into the nucleus, where they associate with a monomer of IRF-9 and form the interferon stimulated gene factor 3 (ISGF3) heterodimer. The ISGF3 heterodimer binds to the IFN stimulated response element (ISRE), which is present in the promoters of most Interferon Stimulated Genes (ISG), and stimulate transcription of these ISGs, such as OAS, PKR, Mx and PML (Haller et al., 2006, **fig. 3**). These ISGs exert their antiviral effects at different stages in the virus life cycle. They have been found to inhibit viral RNA transcription and translation, to disturb post-translational modification of viral proteins and to alter the viral packaging (Chopy et. al., 2011).

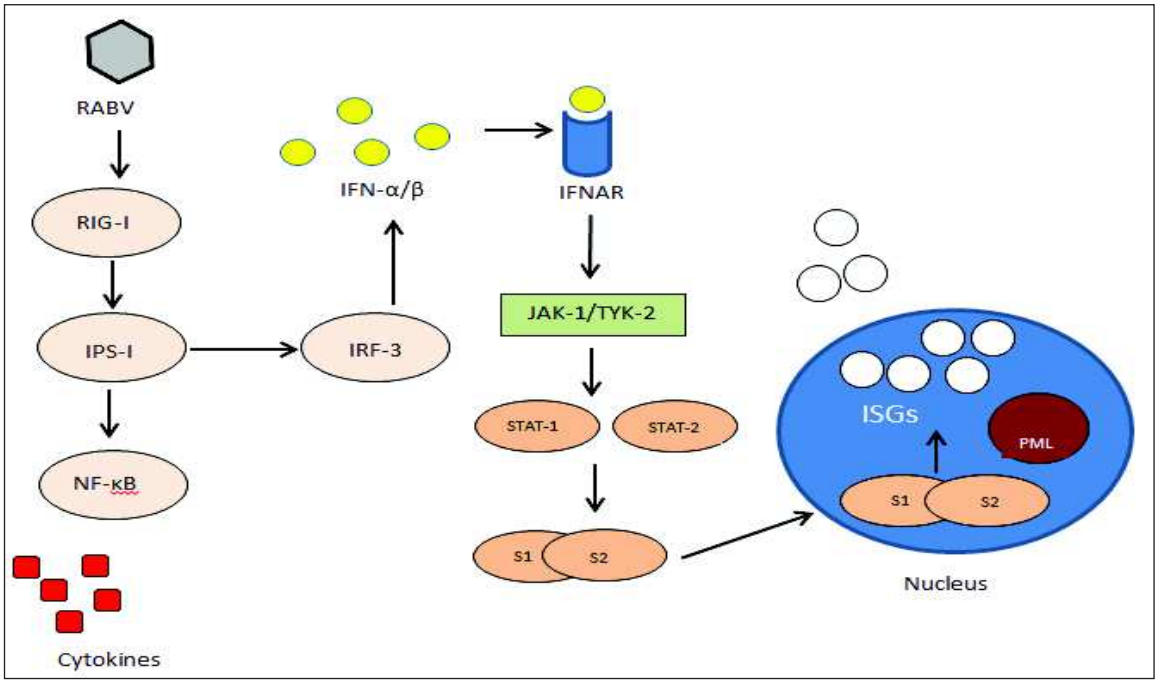

Figure 3: *Based on Haller et al., 2006.* Viral induction of interferon response

The virus is recognized by RIG-I, leading to activation and interaction with IPS-1. IPS-1 phosphorylates IRF-3 and activates NFκB, which are responsible for the expression of type-I IFN and inflammatory cytokines, respectively. The produced type-I IFN binds to IFNAR which leads to recruitment of JAK-1 and TYK-2. These kinases phosphorylate STAT-1 and -2 which form complexes that are translocated into the nucleus. In the nucleus, they induce transcription of ISGs.

3.2.3 Suppression of interferon response

Viruses would not be successful pathogens if they had not developed mechanisms that allow them to suppress the interferon production, signalling and antiviral activity of proteins produced. Studies show that the capability of a virus to antagonize the IFN response is related to the virulence and pathogenicity (Haller et al., 2006).

There are several ways in which a virus can evade the interferon response, and to make evasion sufficient, the virus might combine two or more of these mechanisms. Viruses often block interferon production specifically, and some antagonize antiviral interferon-induced proteins like PKR (Randall and Goodbourn, 2008)

The amount of interferons produced depends on: 1) the type and amount of interferon inducer synthesised as a response to the viral infection; 2) the potential interferon inhibitors the virus produces; 3) the type of cell that is being infected (Randall and Goodbourn, 2008).

3.2.4 Rabies virus suppression of interferon response

RABV replicates in neurons and neurons are able to produce interferons in response to infection, so it appears that the rabies virus' ability to block the interferon system might be important regarding its virulence.

The P-protein has been reported to be responsible for evasion of RABV of the interferon response in several ways (Niu et al., 2013). Experimental studies (Ito et al., 2010) comparing pathogenicity of three rabies virus strains; differing in the P protein, suggest that RABV P protein is a key regulator of viral pathogenicity. By comparing the propagation of the different strains in mouse brain, it also suggests that host innate immunity plays a role in the mechanism by which the P-gene determines pathogenicity. The study includes several experiments with neuroblastoma cells infected with the different strains and treated differently with interferons. The results indicated that the P protein determines the virus' sensitivity to the interferon response.

P protein also appeared to be responsible for blocking of the IFN-α-induced ISRE activity, and for inhibiting the interferon-induced translocation of STATs into the nucleus. The study further showed that the strain with reduced P protein expression inhibited the STAT nuclear

translocation less efficiently, indicating that the inhibitory effect of the P protein on the STAT nuclear translocation is correlated with viral pathogenicity (Ito et al., 2010).

Other studies also emphasise the key role of P protein in the inhibition of reactive interferon-activation: P protein interacts with STAT-1 (Niu et al., 2013) and forms a complex. The P protein-STAT1 interaction does not block the activation of STAT1, but rather blocks the translocation of the phosphorylated STAT-1 and STAT-2 into the nucleus, and in turn prevents the activation of ISGs' expression (Randall and Goodbourn, 2008, **fig. 4**). According to Brzózka et al. (2005) the key factor for initiating an IFN response is IRF-3, which is expressed in the cytoplasm of most cell types (Brzózka et al., 2005). RABV P protein inhibits interferon expression by blocking the phosphorylation of IRF-3 (Niu et al., 2013).

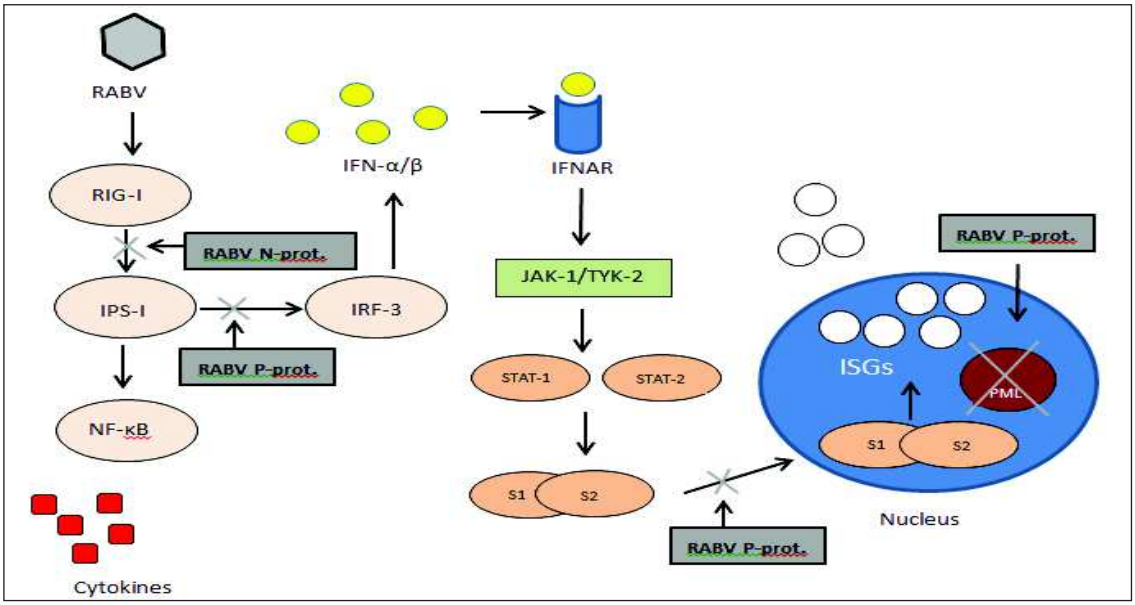

Figure 4: (*Based on Haller, 2006 and Randall and Goodbourn, 2008).* Rabies virus inhibition of interferon response. RABV N protein limits RIG-I signalling, the P protein inhibits IRF-3 phosphorylation, blocks the STAT1 translocation into the nucleus, and in the cytoplasm it sequesters an antiviral protein, the promyelolytic leukemia protein (Chopy et. al., 2011).

3.3 Ribavirin

Ribavirin is a non-toxic guanosine analogue with broad-spectrum antiviral activity. Ribavirin was one of the drugs included in the Milwaukee protocol that was used in the only non-vaccinated patient that has ever survived rabies after onset of clinical signs (Fooks et al., 2014; Appolináriol et al. 2013). Because ribavirin was given in combination with other drugs, it is difficult to say how effective ribavirin actually was, and the treatment regimen has never been successfully repeated (Jackson, 2010; (Appolináriol et al. 2013).

Ribavirin was originally only approved for the treatment of Respiratory Syncytial Virus infections in children, but it has also been used in the treatment of other viral diseases such as influenza virus and hepatitis C (HCV) infection. Ribavirin is now a part of the standard treatment protocol of chronic HCV infection, where the effect of ribavirin alone is insignificant in regards to RNA levels, but the results when used in combination with IFN-α are excellent (Te et al., 2007). The mechanism of this enhanced effect is not well understood.

In addition to the antiviral properties, ribavirin also has immunomodulatory effects. During the initial states of viral infections, there is a T-helper-1 response: T-cells that produce type 1 cytokines like interleukin-2, IFN-γ and TNF-α are activated attempting to clear the virus. Subsequently, other T-cells that produce type 2 cytokines such as IL-4, IL-5, and IL-10 are activated to stimulate the humoral response. Ribavirin has been demonstrated to have the immunomodulatory effect of shifting a Th2 response in favour of a Th1 phenotype (Te et al., 2007). Ribavirin also stimulates the expression of ISGs with antiviral properties (Zhang et al., 2003).

Ribavirin is converted into ribavirin monophosphate by adenosine kinase. It is then converted into dephosphorylated- and triphosphorylated forms by nucleoside monophosphate and diphosphate kinases. Ribavirin triphosphate (RTP) is the form that dominates in most cells (Te et al., 2007).

RMP inhibits inosine monophosphate dehydrogenase (IMPDH), causing a decrease in the intracellular concentration of GTP. This decrease interferes with the synthesis of viral proteins and the replication of viral genomes (Crotty et al., 2000).

However, inhibition of IMPDH is not sufficient for antiviral activity, and so is not the main mechanism of action.

The suggested main mechanism of action of ribavirin is its mutagenic activity. Ribavirin is a competitive inhibitor of viral RNA polymerase. Its incorporation is mutagenic, and the antiviral activity directly correlates with this mutagenic activity. Ribavirin forces the virus into "error catastrophe" (Crotty et al., 2000) or lethal mutagenesis. When the RTP is bound to the nucleotide binding site of the RNA polymerase, it blocks the binding of the correct nucleotides. This leads to the production of defective virions and reduced virus replication (Te et al., 2007, **fig. 5**).

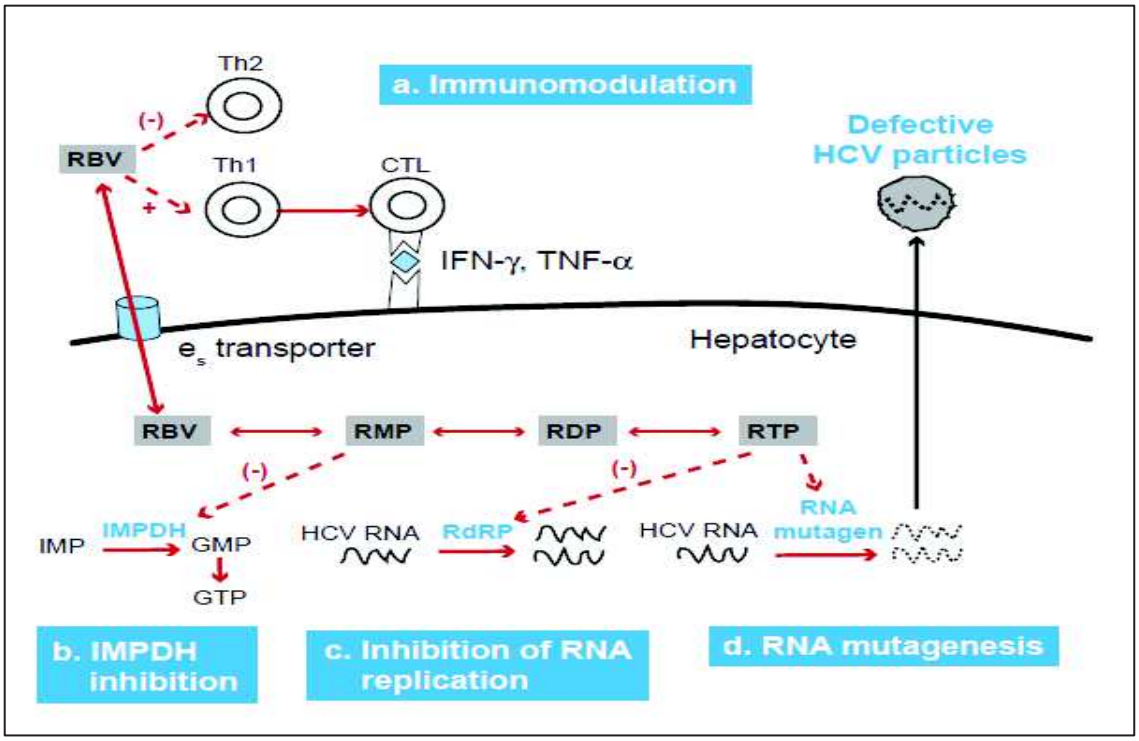

Figure 5: (*Helen S. Te, 2007)*. Proposed sites of ribavirin action against hepatitis C virus (HCV): a) Induction of a shift from Th2 to Th1 immune response, b) Inhibition of IMPDH leading to GTP pool depletion, c) Direct inhibition of HCV replication, and (d) induction of mutagenesis, leading to production of defective viral particles

3.3.1 Ribavirin effect on rabies virus

A study on ribavirin effect on rabies virus infection *in vitro* and *in vivo* (Appolinário et al. 2013) determined that the effect of ribavirin is cell type dependent. It compared the effect of ribavirin treatment in fibroblasts cells and neuroblastoma cells by analysing the Negri-body formation. The results showed that Negri body formation in fibroblast cells treated with ribavirin was drastically decreased. Neuroblastoma cells still showed signs of infection, even though most of the ribavirin-treated-infected cells had smaller Negri bodies compared to the non-treated cells. Ribavirin impairs RABV-replication and transcription of viral genes in both fibroblast and neuroblastoma cells, but less efficiently in neuroblastoma cells (Appolinário et al. 2013).

Oral treatment with ribavirin triggers a transient innate immune response in the brain in the absence of infection: it leads to an increase in mRNA expression of OAS-1 gene, chemokine CCL-2, inflammatory cytokines IL-6 and TNF-α and that of IFN-β. This indicates that when given orally, ribavirin can penetrate into the brain. This possibility has also been supported by the presence of ribavirin in the cerebrospinal fluid of animals that have received long term oral treatment with ribavirin (Appolinário et al. 2013).

Trying to reach the same effect in an experimental model of rabies in mice, the oral ribavirin treatment fails. There is no difference between infected animals treated or non-treated, regardless of dose and duration of treatment (Appolinário et al. 2013). This indicates that oral treatment with ribavirin has no effect on RABV neuroinvasiveness, and is suggesting that appropriate dosage or route of administration has not been determined yet.

3.4 Favipiravir

Favipiravir, also known as T-705 is a novel antiviral drug, first published in 2002 by Toyama Chemicals, Japan (Sangawa et al., 2013). Favipiravir shows antiviral activity against a broad spectrum of RNA viruses such as Influenza, Flaviviruses such as West Nile Virus and Yellow Fever Virus, Arenaviruses, Bunyaviruses and more. Importantly, several viruses resistant to both the antiviral drugs oseltamivir and amantadine have been shown to be susceptible to favipiravir (Baranovich et al. 2013).

Favipiravir is a prodrug. Cellular enzymes phosophoribosylate favipiravir into its active form: favipiravir-ribofuranosyl-5´-triphosphate (favipiravir-RTP) (Furuta et al., 2013). The mechanism of action is not fully understood, but it is suggested that, similar to ribavirin, it is a nucleotide analogue that targets and inhibits RdRP, and causes lethal mutagenesis upon incorporation into the virus RNA (Baranovich et al. 2013). Its antiviral effect is attenuated by the addition of nucleic acids, suggesting that the viral RNA polymerase mistakes favipiravir-RTP for a purine nucleotide (**fig. 6**).

An important feature of favipiravir is that it does not inhibit host DNA/RNA synthesis and exerts its antiviral effect without significant toxicity to the host (Furuta et al., 2013). To investigate the impact of favipiravir on host cell and viral nucleotide metabolism, the inhibitory activity of favipiravir-RTP has been examined against human RNA and DNA polymerases and viral RdRP. Favipiravir-RTP inhibited the RdRP of influenza virus at a 50% inhibitory concentration (IC_{50}) of 0.341 μM, whereas IC50 values for human DNA polymerase α, β or γ and human RNA polymerase-II were found >1000 μM and 905 μM, respectively (Takahashi et al., 2011). This means that favipiravir is 2650 times more selective for the influenza virus RdRP than human DNA/RNA-polymerases, consistent with the lack of inhibition of host-cell DNA and RNA synthesis. Favipiravir has been administered to over 1400 patients in clinical trials all over the world and has shown a very good safety record (Furuta et al., 2013).

Studies that investigated the effect of favipiravir treatment at different times during the virus replication cycle using influenza virus, revealed that this antiviral drug exerts its effect by interfering with viral replication. The mechanism of inhibition was also investigated by

simultaneous addition of nucleosides. This addition of purine nucleosides reduced the antiviral activity, while pyrimidine bases did not. The results indicate that favipiravir is a pseudo-purine (Mendenhall et al., 2011a).

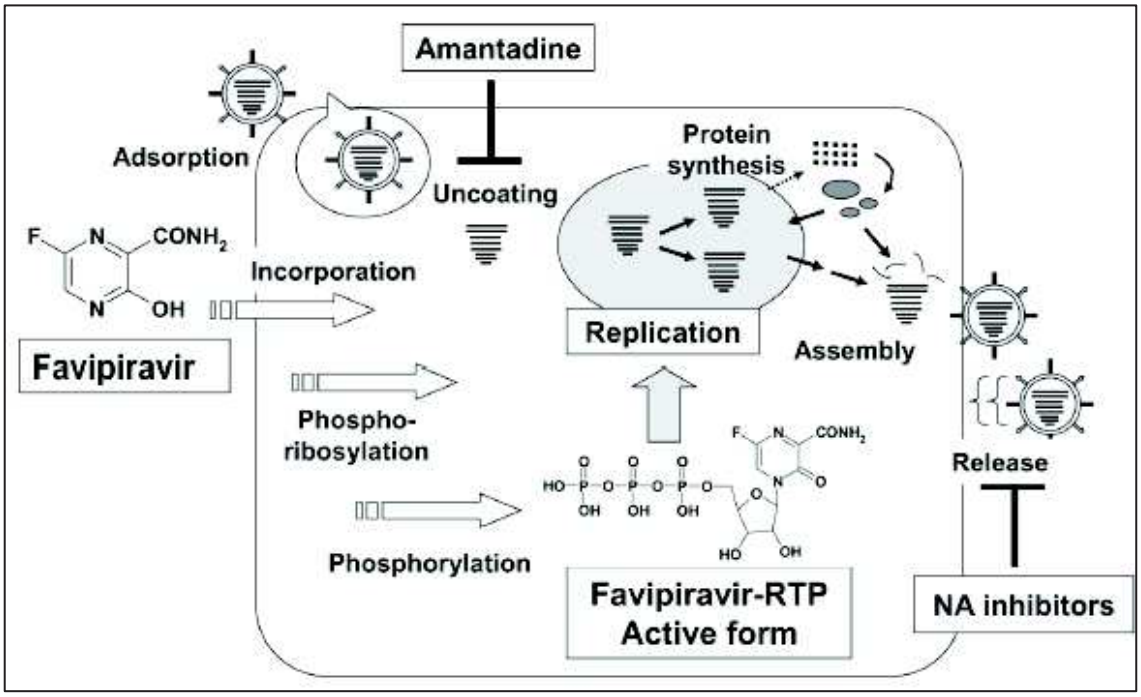

Figure 6: *(Furuta et al., 2013).* Mechanism of action of favipiravir. Favipiravir is converted to Favipiravir-RTP by host cell enzymes and selectively inhibits the activity of the influenza viral RNA polymerase.
The figure also shows the place of action of other antiviral drugs (amantadine and NA inhibitors).

With its unique mechanism of action and broad range of antiviral activity, favipiravir is a promising drug candidate for many RNA viral diseases for which there are no approved therapies.

4 Materials and methods

4.1 Cell lines, virus and reagents

Mouse neuroblastoma (N2A) cell line was obtained from the Viroscience Lab of Erasmus Medical Center (Rotterdam, NL). Karen L. Mansfield (Animal & Plant Health Agency, Weybridge, UK) kindly provided the fixed RABV strain for in vitro studies, CVS-11 (Challenge Virus Standard) in a titre of 10^5 $TCID_{50}$/ml. Each experiment where live virus was present was performed in a BSL (Biological Safety Level)-3 laboratory operated by the Institute for Veterinary Medical Research (Centre for Agricultural Research, Hungarian Academy of Science, Budapest).

Recombinant mouse interferon (IFN)-alpha and -beta were purchased from Merck (Darmstadt, Germany). Ribavirin and favipiravir (T-705) was kindly provided by Dirk Jochmans (University of Leuven, B). The FITC (fluorescein isothiocyanate)-conjugated monoclonal globulin against the RABV nucleoprotein (NP) was obtained from Fujirebio Diagnostics, Inc. (Malvern, USA).

4.2 Culture of N2A cells

The N2A cells were stored at -80˚C, and then they were grown in 75 cm^2 cells culture flasks (Sarsedt AG & CO., Nümbrecht, D) at 37˚C and 5% CO_2. The medium was Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/l glucose with ultraglutamine (Lonza, Walkersville, USA) supplemented with 10% foetal calf serum (FCS) and appropriate antibiotics/antimycotics. Once the monolayer was confluent, trypsin-EDTA solution was added to create a cell suspension. Later, the suspension was adjusted with DMEM to a cell count of 4×10^5 cells/ml.

100 µl cell suspension was added to each well of a 96-well cell culture microplate with advanced polymer coating (Greiner Bio-One, Kremsmünster, A). The plates underwent an incubation period of 24 hours at 37˚C and 5% CO_2 prior to the experiments.

4.3 Post-infection antiviral assay

Every candidate antiviral substance was diluted to four different concentrations with 10-fold serial dilution. The highest concentration was selected based on the results of a cytotoxicity assay (commercial kit by Roche, Basel, Switzerland) on N2A cells (measuring lactate-dehydrogenase activity) completed for each substance prior to experiments. For the compound combination treatment experiments, the concentrations were chosen according to the results of the antecedent experiments involving single compounds. The different concentrations of the different compounds and their combinations are shown in **tables 1-2**. DMEM containing 2% FCS was used to dilute the stock solution of the drugs to the desired concentration.

Table 1: Dilutions of test compounds used in post-infection antiviral assay

Compound	**Stock solution**	**Test concentrations**			
IFN-α	1000 IU/ml (in PBS)	10 IU/ml	1 IU/ml	10 IU/ml	10 IU/ml
IFN-β	1000 IU/ml (in PBS)	10 IU/ml	1 IU/ml	10 IU/ml	10 IU/ml
IFN-α and -β	500-500 IU/ml (in PBS)	5-5 IU/ml	0.5-0.5 IU/ml	0.05-0.05 IU/ml	0.005-0.005 IU/ml
Ribavirin	10 mg/mL (in DMSO)	10 µg/mL	1 µg/mL	0.1 µg/mL	0.01 µg/mL
T705 - favipiravir	10 mg/mL (in DMSO)	10 µg/mL	1 µg/mL	0.1 µg/mL	0.01 µg/mL

Table 2: Combinations of compounds used in post-infection antiviral assay

Combination	**Test concentrations**			
IFN-β ribavirin	1 IU/ml 10 µg/ml	1 IU/ml 1 µg/ml	0,1 IU/ml 10 µg/ml	0,1 IU/ml 1 µg/ml
IFN-β T705	1 IU/ml 10 µg/ml	1 IU/ml 1 µg/ml	0,1 IU/ml 10 µg/ml	0,1 IU/ml 1 µg/ml
ribavirin T705	10 µg/ml 10 µg/ml	10 µg/ml 1 µg/ml	1 µg/ml 10 µg/ml	1 µg/ml 1 µg/ml

The cell culture microplates were incubated for 24 hours (as described in **4.2**) before the medium was discarded and replaced to 50µl plain DMEM (without FCS) containing 5000 $TCID_{50}$ of CVS-11 virus (MOI=0,125). Concentration of the challenge virus suspension was adjusted to this level to ensure that close to 100% of the cells in a well were infected after 48 hours.

In case of the negative control wells only DMEM was added without virus. The plates were incubated for 1 hour at 37°C and 5% CO_2 in order to let the virus particles adsorb on N2A cells. After the 1 hour incubation, the virus-containing DMEM was discarded, and the antiviral solutions were transferred to the appropriate wells according to the actual plate design. The plates were placed into thermostat for a 48 hours incubation period.

After the 48 hours, the supernatant from each well was collected and immediately stored at -80°C for the subsequent studies; titration – FFA; and qRT-PCR. The plates were fixed with 80% acetone and stained with fluorescent antibody conjugate (**4.4.**), and the percentage of cells with fluorescent Negri-bodies was estimated in each well, using an inverted fluorescent microscope. Two independent readers visually assessed the wells and the mean of the findings was used as the result.

4.4 Fixation and fluorescent staining of RABV-infected cell cultures

80% acetone was used (diluted with distilled water) for the fixation of the microplates with RABV-infected cells. The wells were completely filled with acetone, and were incubated at room temperature for 20 minutes. Subsequently, the acetone was discarded and the plates were left to air dry in a Class-2 biological safety cabinet with laminar air-flow.

After fixation, the cells were stained with fluorescent-labelled conjugate of anti-rabies monoclonal globulin (Fujirebio Diagnostics, Inc., Malvern, USA). The antibody binds to the N protein of RABV, and is conjugated with fluorescein-isothiocyanate. 50µl of conjugate was added to each well, and the plates were incubated for 30 minutes in darkness. Lastly, the conjugate was removed, and the cells were washed twice with PBS (phosphate buffered saline). Once the staining was completed, the plates were evaluated using an inverted fluorescent microscope. In the case of RABV-infected cells, striking apple-green fluorescence could be observed

4.5 Fluorescent focus assay

The titration method called FFA (fluorescent focus assay) was used to evaluate the level of viral replication in the presence (or absence) of different antiviral compounds and/or their combinations. For this analysis, the supernatants that were removed from the cell cultures after 48 hours incubation with the compounds (**4.3**) were assessed to determine the viral titres.

Multiple 96-well plates with N2A cell cultures were prepared (as described **4.2.**) and incubated for 24 h. On a same number of other plates, 10-fold serial dilutions were performed from supernatant samples of the initial antiviral plate (the plate used for post-infection studies, see **4.3.)**. The supernatants were titrated in quadruplicates (four parallel dilutions of one sample). The diluent was plain DMEM; without FCS. The medium was removed from the cell cultures, and then 50µl from each well of plates with the dilutions were transferred to the corresponding well of the cell culture plate. Following a 1 hour long incubation for virus adsorption, 200µl of cell culturing medium (DMEM with 5% FCS) was added to each well, and the plates were placed into thermostat for 48 hours.

After the 48 hours, the supernatant was removed from the cells, and the plates were fixed and stained according to the protocol described before (see **4.4.)**. When evaluating the FFA titration plate a well with only one fluorescent cell within was considered to be positive. Viral titres of the supernatant samples were calculated using the Spearman-Karber formula (see **4.7**).

4.6 RNA extraction and real-time reverse-transcription PCR (qRT-PCR)

Real-time reverse-transcription polymerase chain reaction (qRT-PCR) was performed to determine the amount of viral RNA using the same supernatant samples from the original antiviral plate.

For RNA extraction a commercial kit was used (QIAamp Viral RNA Mini Kit by Qiagen, Hilden, D) with the manufacturer's instructions:
First, 560µl of lysis buffer (AVL) was added to 140µl of each supernatant sample. After 10 minutes of incubation at room temperature 560µl of 100% ethanol was added in order to precipitate the nucleic acid. The mixture was transferred into a silica filter tube, and the tube was centrifuged for 1 minute at 6000×g. To filter the whole amount of mixture (2×630µl), this last step was repeated. After this, there were two washing steps: washing buffer AW1 and AW2 was added to the filter tube, and after adding each buffer, the tube was centrifuged (1 minute at 6000×g; 3 minutes at 20000×g). An additional centrifugation was included; 1 minute at 20000×g to get rid of any traces of buffers to leave only pure RNA on the filter. Lastly, 60µl of elution buffer was added to the filter tube to wash off the purified RNA into a micro-centrifuge tube which was centrifuged for 1 minute at 6000×g. The extracted RNA was kept in freezer (-70°C) until further use.

To quantify viral RNA in the samples extracted with the method described above a single-tube qRT-PCR test was performed using Qiagen 1-step RT-PCR kit (Qiagen, Hilden, D). In a 1-step RT-PCR approach reverse transcription (RT) and following amplification (qPCR) of cDNA are performed in the same tube, using an enzyme mixture that is included in the kit (containing both reverse transcriptase and DNA-polymerase enzyme). Compared to a two-step approach, this technique has higher reproducibility and lower risk of contamination because less sample processing is required.

The total volume of reaction mixture for one sample was 25µl. **Table 3** shows the amount of ingredients. Reaction mixture containing RNA extracted from CVS-11 stock was used as a positive control. For the negative control, nuclease-free water was added to the mixture instead of template RNA. **Table 4** shows the sequence of primers and the probe specific to RABV.

Table 3: Components of the reaction mixture prepared for qRT-PCR

Reagent	**Volume (µL) for 1 sample**
Nuclease-free H_2O	12.15
Qiagen 1-step RT-PCR buffer (5×)	5
$MgCl_2$ (25mM; Applied Biosystems)	1.25
dNTP (10mM, Qiagen)	1
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Probe (10 µM)	0.5
RNAse inhibitor	0.1
Qiagen RT PCR enzyme mix	1
Template RNA	2

Table 4: Primer and probe sequences used in the studies

Primer/Probe		**Length**	**Melting temp. (°C)**	**Position***	**Sequence** (5' – 3')**
Forward	JW12	19	49	55-73	ATGTAACACCYCTACAATG
Reverse	N165-146	20	53	165-146	GCAGGGTAYTTRTACTCATA
Probe***	LysGT1	29	62	81-109	ACAAGATTGTATTCAAAGTCA ATAATCAG

* Positions based on genome of the Pasteur virus strain (accession no. M13215; Tordo et al, 1988)
** Y: pyrimidin base (thymine or cytosine); R: purine base (adenine or guanine)
*** Probe labelling: 5'-6FAM 3'-TAMRA

The amplification was performed at Institute for Veterinary Medical Research (Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest) using an Applied Biosystems StepOne Plus real-time PCR machine (Thermo Fisher Scientific, Inc, Waltham, USA). **Table 5** shows the protocol that was followed. Fluorescence emission was detected at the end of each extension step. Viral RNA content was calculated from the C_t-values as described later (see **4.7)**.

Table 5: qRT-PCR protocol

Step	**^{0}C**	**Duration**	
RT	50	30 min	
Activation	95	15 min	
Denaturation	95	20 sec	repeated 40×
Annealing	55	30 sec	
Extension	72	30 sec	

4.7 Data analysis

In FFA experiments viral titres were calculated with the Spearman-Kärber (Spearman, 1908; Kärber, 1931) formula and expressed in $TCID_{50}$/ml. Using this method the amount of infectious virus in a cell culture supernatant can be calculated by determining the dilution that causes cytopathic changes in 50% of the inoculated cell cultures. This dilution is called the 50% tissue culture infectious dose endpoint ($TCID_{50}$). RABV is not a cytopathic virus, so in this case, a fluorescent labelled monoclonal antibody was applied to reveal infected cell cultures with the use of a fluorescent microscope.
For calculation of $TCID_{50}$ the following formula can be used:

$$\log (TCID_{50}) = x + 1/2d - (d*\Sigma r)/n$$

X means the 10-base logarithm of the highest dilution applied (eg. for a 1000-fold dilution x=3); ***d*** indicates the 10-base logarithm of the dilution factor (in most cases, when a 10-fold serial dilution is used, d=1); ***Σr*** is the overall number of negative wells in the assay, whereas ***n*** means the number of repetitions for each dilution (in all studies reported in this thesis work, titrations were performed in quadruplicates, therefore n=4).

The raw results of qRT-PCR experiments are threshold cycle (C_t) values. C_t means the number of cycle where the fluorescence generated by probe-labelled cDNA crosses the threshold line (where the signal is significantly higher than the background fluorescence). The exponential phase of amplification starts at this point. For higher number of RNA copies present in the sample lower C_t-value will be determined, due to the fact that the amplification becomes exponential at an earlier cycle. A 6-point calibration curve was constructed based on C_t values of a 10-fold serial diluted CVS-11 viral RNA with known titre. Based on linear regression of this calibration curve viral titres of the supernatant samples were calculated from corresponding C_t values. Every sample was assayed in duplicate, and the means of their C_t-values were used as result.

The results are presented as mean ± SEM of three different experiments (n=3) for FFA test and semi-quantitative assessment of fluorescence (**4.3**.). Data was statistically analysed using the R Software Package (version 3.1.2). Student's t-test was applied for comparison of experimental groups at a significance level of 5% (p=0.05).

5 Results

5.1 Single compound treatment

5.1.1 IFN-α

The results of the semi-quantitative analysis can be seen in **fig. 7.** The effect of the four different doses is compared to the positive control. 10 IU/ml reduced the percentage of infected cells to 0.2333%. 1 IU/ml reduced the percentage to 31.6667%, 0.1 IU/ml reduced the percentage to 66.6667% and 0.01 IU/ml reduced it to 96.6667%.

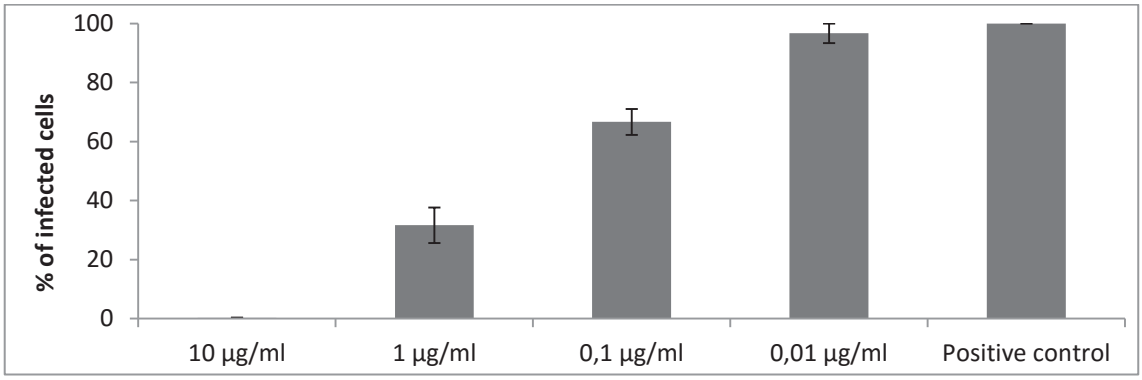

Figure 7: Semiquantitative analysis of IFN-α treatment

The results of the FFA and the qRT-PCR are presented in **fig. 8**. The results of the two methods are compared. For both methods, the effects of the four different doses are compared to the positive control. The FFA results show a 3.5 fold decrease from the positive control with 10 IU/ml. 1 IU/ml caused almost a 2 fold decrease in viral titres, and both 0.1 and 0,01 IU/ml caused a 1 fold decrease.

The qRT-PCR shows that 10 IU/ml caused over a 1 fold decrease in virus quantity. The results of the qRT-PCR show that 1 IU/ml, 0.1 IU/ml and 0.01 IU/ml had no observable effect. The lowest dose well had a slightly higher quantity than the positive control.

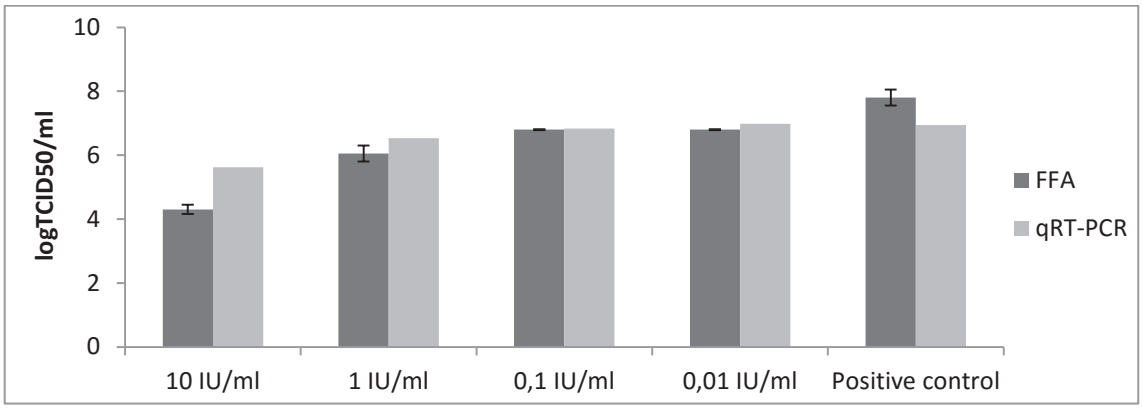

Figure 8: FFA and qRT-PCR analysis of effect of IFN-α treatment

5.1.2 IFN-β

The results of the semi-quantitative analysis can be seen in **fig. 9**. The effect of the four doses is compared to the positive control. 10 IU/ml reduced the percentage of infected cells to 0.2333 %. 1 IU/ml reduced the percentage to 11.00%, 0.1 IU/ml reduced it to 55.00% and 0.01 IU/ml reduced the percentage to 83.3333%.

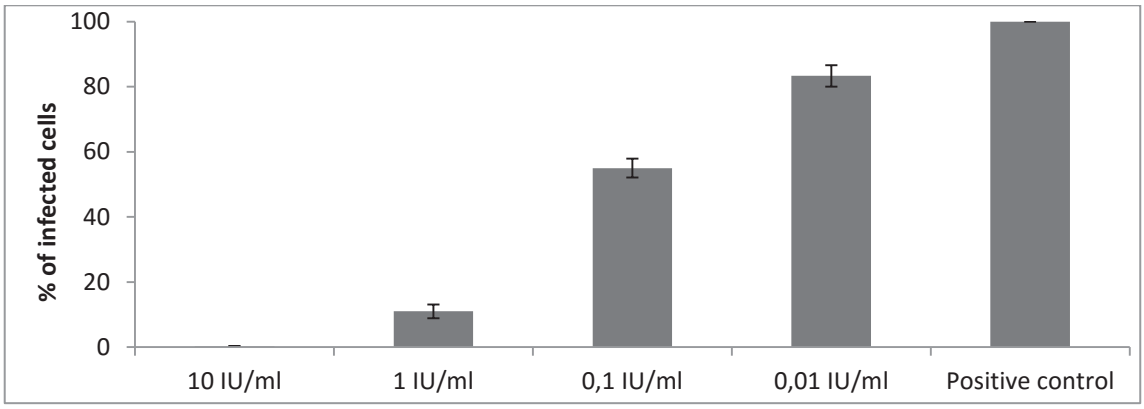

Figure 9: Semiquantitative analysis of IFN-β treatment

The results of the FFA- and qRT-PCR analysis can be seen in **fig. 10**. The results of the two methods are compared, and for both methods, the effects of the four different doses are compared to the positive control. FFA shows that 10 IU/ml caused almost a 5 fold decrease in the viral titre compared to the positive control. 1 IU/ml decreased the viral titre by almost a 4 fold, and 0.1 IU/ml caused more than a 1 fold decrease. According to the FFA results, 0.01 IU/ml caused no decrease on viral titre. PCR shows the same trend, but a smaller decrease in virus quantity. The results for 10 IU/ml show a 2.5 fold decrease, 1 IU/ml almost a 2 fold

decrease, 0.1 IU/ml less than 1 fold decrease, and 0.01 IU/ml caused no reduction. The PCR results for 0.01 IU/ml actually shows slightly more virus than the positive control.

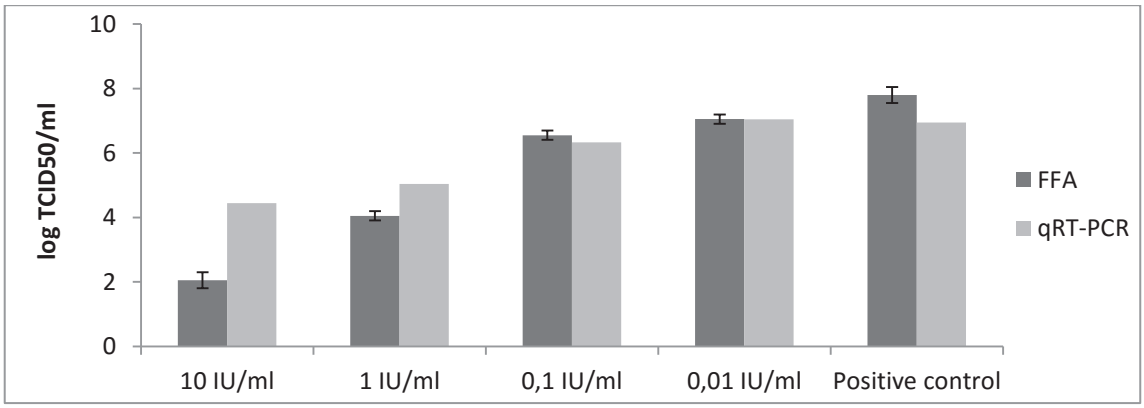

Figure 10: FFA and qRT-PCR analysis of effect of IFN-β treatment

5.1.3 IFN-α and IFN-β combination

The IFN-α and IFN-β combination treatment was done together with the single compound treatments. This was done in order to find out which interferon had a better effect, so that only the best interferon would be included in the subsequent combination treatments. This way the number of possible combinations could be reduced to make the experiments manageable in terms of working hours and consumable requirements.

Fig. 11 shows the results of the semi-quantitative analysis of the IFN-α and IFN-β combination treatment. The effect of the four doses is compared to the positive control.

Fig. 12 shows the comparison of the results of the FFA and qRT-PCR analysis of the IFN-α and IFN-β combination treatment.

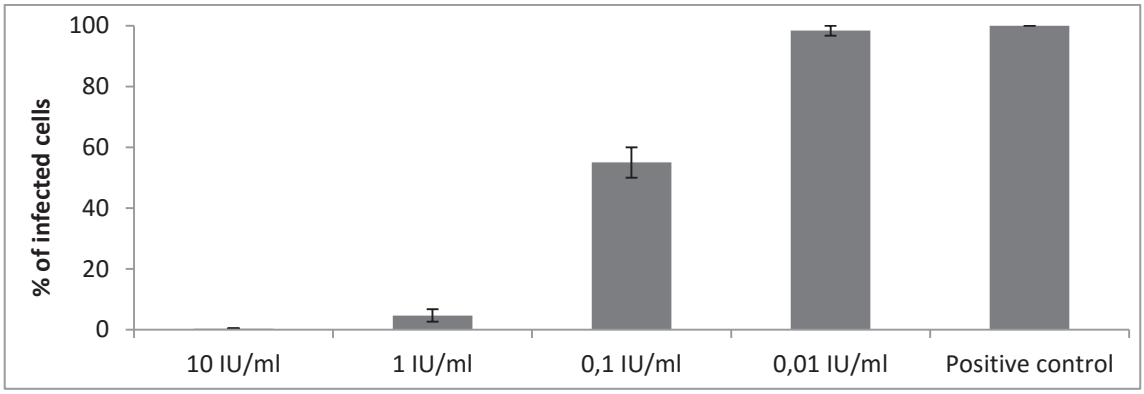

Figure 11: Semi-quantitative analysis of IFN-α and IFN-β combination treatment

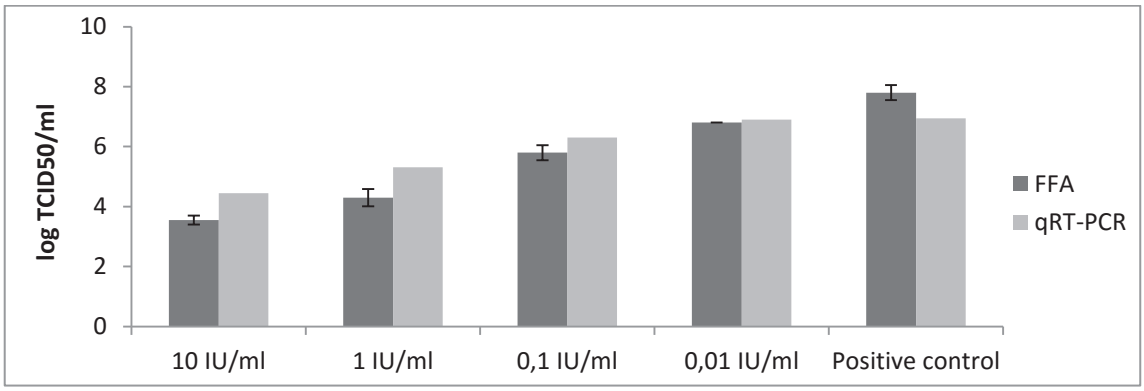

Figure 12: FFA and qRT-PCR analysis of effect of IFN-α and IFN-β combination treatment

Fig. 13 shows the comparison of the effect of single IFN-β treatment and the effect of the treatment with the combination of IFN-α and IFN-β. The results show that IFN-β has a better effect than IFN-α, and that IFN-β had almost the same effect on its own as the combination. So in the subsequent combination treatments, only IFN-β was included.

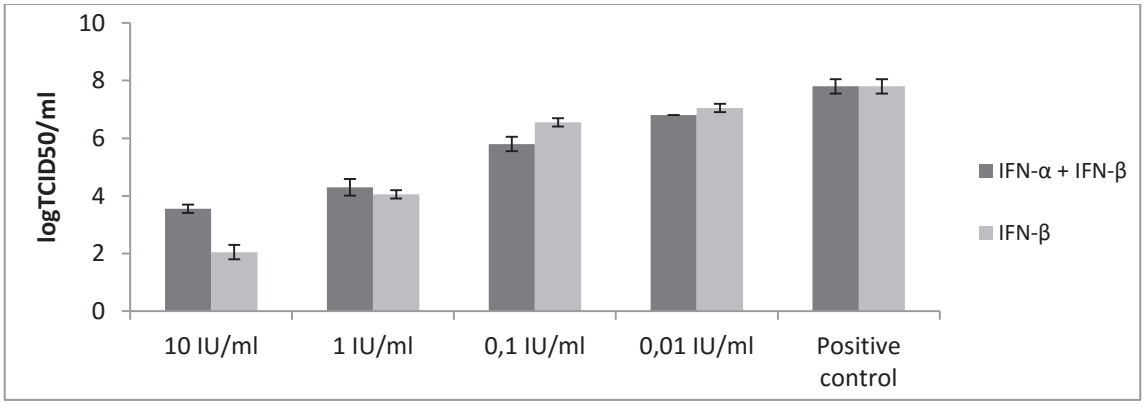

Figure 13: Comparison of FFA results of IFN-β single treatment and IFN-α and IFN-β combination treatment

5.1.4 Ribavirin

The results of the semi-quantitative analysis of ribavirin treatment are presented in **fig. 14**. The effect of the four doses is compared to the positive control. The highest dose of ribavirin; 10 µg/ml, reduced the percentage of infected cells from the positive control of 100% to 8.37%. 1 µg/ml reduced the percentage of infected cells to 73.33%, whereas 0.1 µg/ml reduced it to 93.33% and the lowest dose 0.01 µg/ml did not cause any reduction.

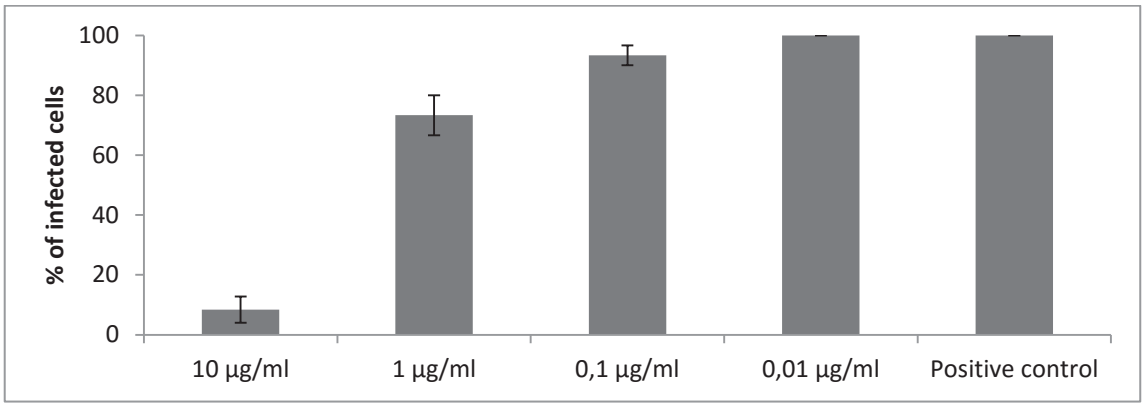

Figure 14: Semi-quantitative analysis of effect of ribavirin treatment

The results of the FFA- and qRT-PCR analysis can be seen in **fig. 15**. The results of the two methods are compared, and for both methods, the effects of the four different doses are compared to the positive control.

The results of the FFA show that 10 µg/ml ribavirin caused a 3.5 fold decrease in viral titre. 1 µg/ml caused a 2 fold decrease, and both 0.1 µg/ml and 0.01 caused less than a 1 fold decrease in viral titre.

The qRT-PCR results show that 10 µg/ml caused just over a 1.5 fold decrease in virus quantity, while 1 µg/ml and 0.1 µg/ml caused less than a 1 fold decrease. The mean of the wells treated with 0.01 µg/ml shows a slightly higher virus quantity than the positive control.

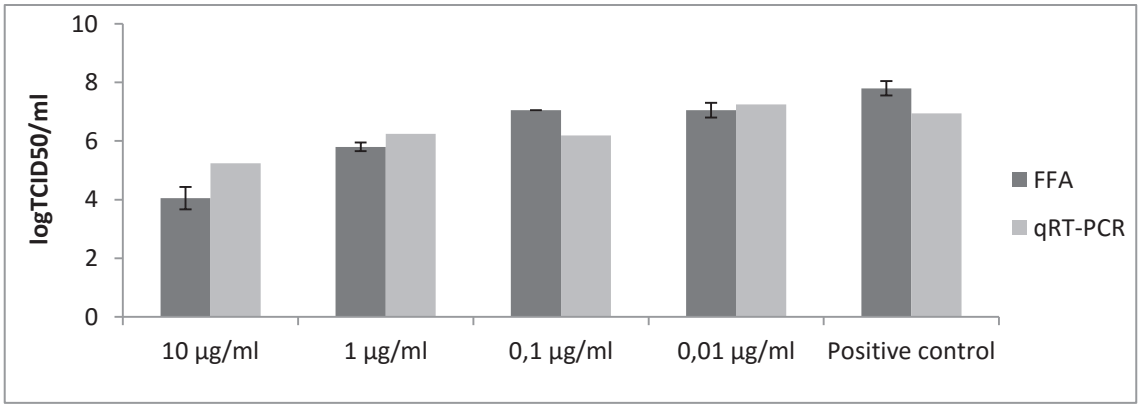

Figure 15: FFA and qRT-PCR analysis of effect of ribavirin treatment

5.1.5 Favipiravir

The results of the semi-quantitative analysis can be seen in **figure 16**. The effect of the four doses is compared to the positive control. 10 µg/ml caused the percentage of infected cells to decrease to 13.33%. 1 µg/ml caused a decrease to 81.67% and 0.1 1 µg/ml caused a decrease in the percentage of infected cells to 95.00%. According to the semi-quantitative analysis 0.01 µg/ml caused no decrease.

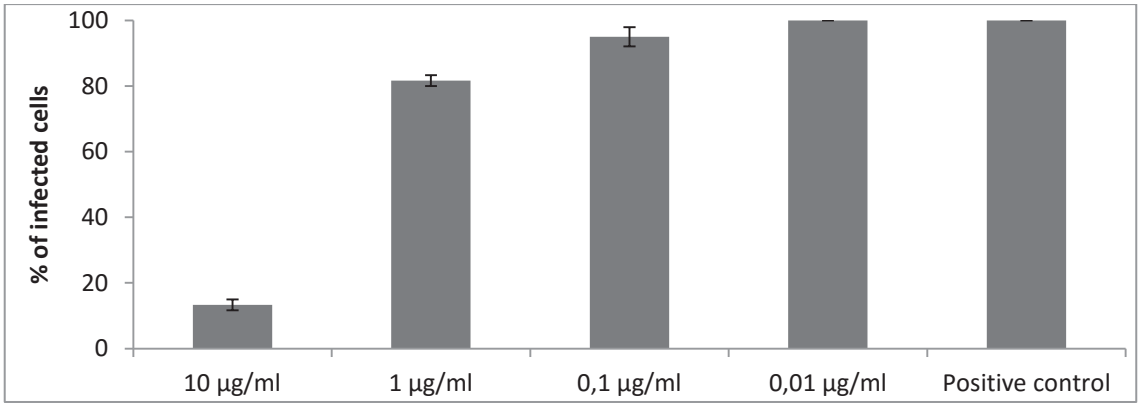

Figure 16: Semi-quantitative analysis of effect of favipiravir treatment

The results of the FFA- and qRT-PCR analysis can be seen in **fig. 17.** The results of the two methods are compared, and for both methods, the effects of the four different doses are compared to the positive control. The results of the FFA analysis show that treatment with 10 µg/ml favipiravir caused almost a 3 fold decrease in viral titre. Treatment with 1 µg/ml caused a 1.5 fold decrease, 0.1 µg/ml caused a 1 fold decrease, while treatment with 0.01 µg/ml had no effect on the viral titre. According to the qRT-PCR analysis, treatment with 10 µg/ml caused only a 1 fold decrease, and the three lower doses had no effect on the virus quantity.

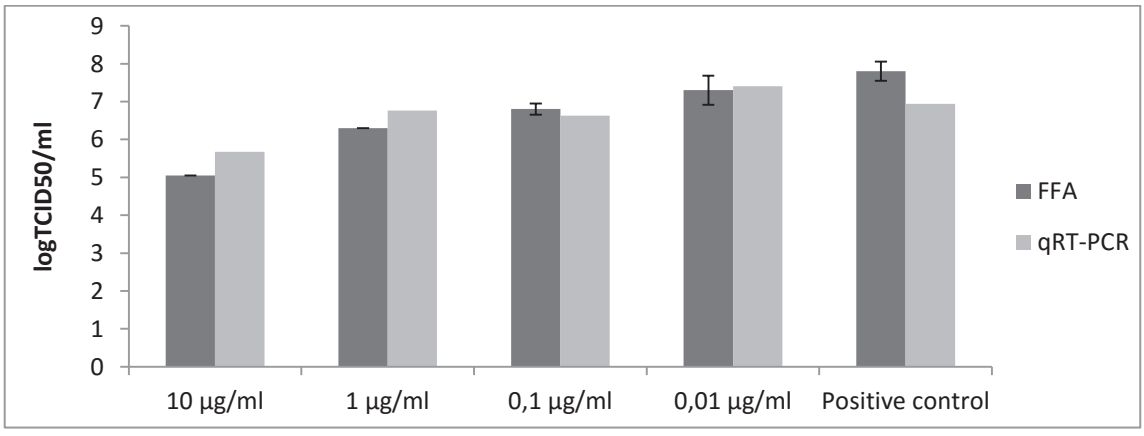

Figure 17: FFA and qRT-PCR analysis of effect of favipiravir treatment

5.2 Combination treatment

All three compound combinations were given at four different doses.

On the horizontal axis of the graphs the different doses are given. The first number in the combination is for the first drug mentioned in the combination name. The unit for IFNβ- is IU/ml, and the unit for ribavirin and favipiravir (T-705) is µg/ml.

Fig. 18, 19 and **20** compare the effect of the different combinations at the different doses. **Fig. 18** shows the results of the semi-quantitative analysis, **fig. 19** shows the results of the FFA analysis and **fig. 20** shows the results of the qRT-PCR.

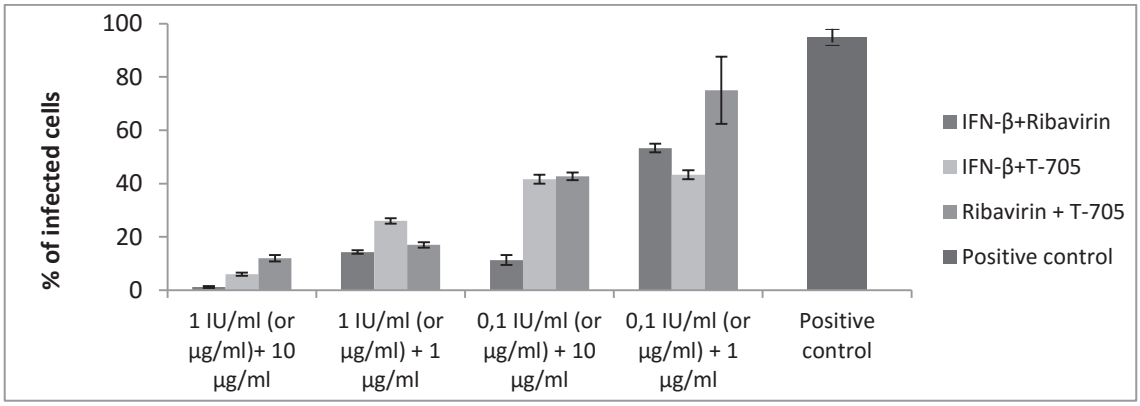

Figure 18: Semi-quantitative analysis of combination treatment

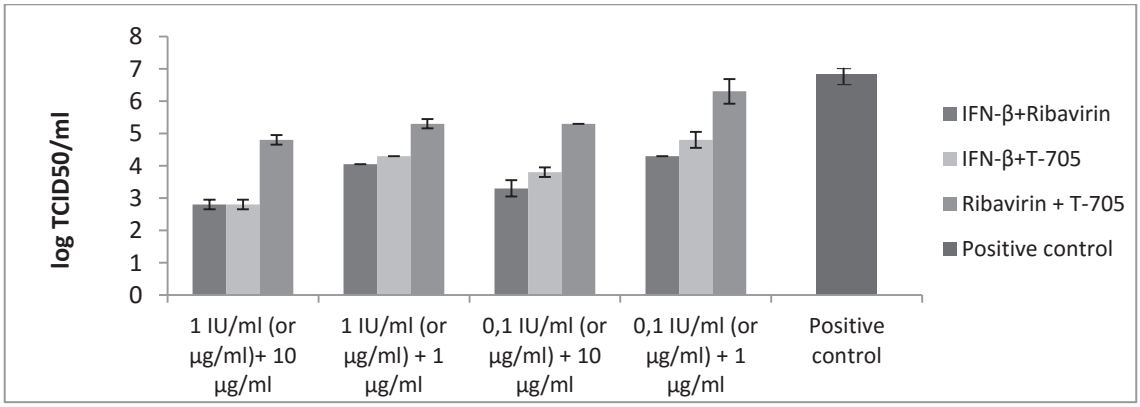

Figure 19: FFA analysis of combination treatment

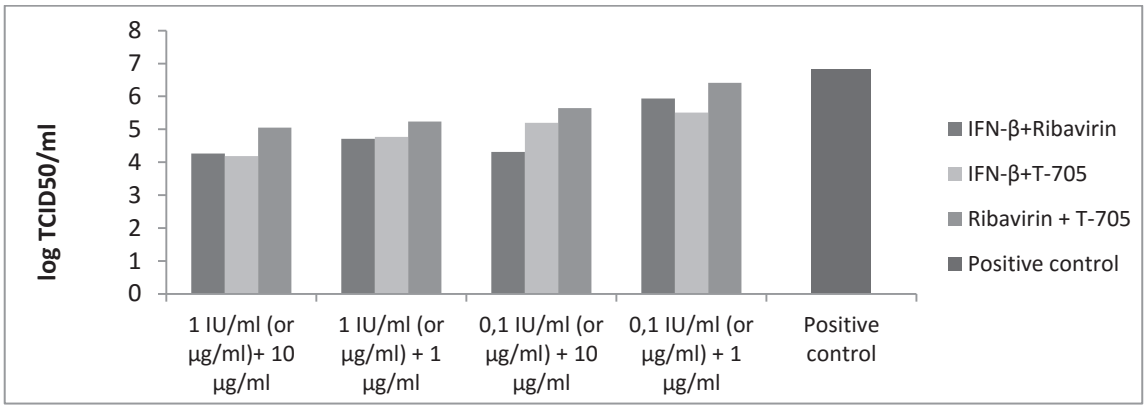

Figure 20: qRT-PCR analysis of combination treatment

5.2.1 IFN-β + ribavirin

The results of the semi-quantitative analysis are presented in **fig. 18**. The results show a reduction of the number of infected cells from 95% in the positive control to 1.2% with the combination of high dose IFN-β and high dose ribavirin. High dose IFN-β and low dose ribavirin reduced the number to 14.33% while low dose IFN-β combined with high dose ribavirin reduced it to 11.33%. The combination of low dose interferon and low dose ribavirin only reduced the number of infected cells to 53.33%.

The results of the FFA analysis (**fig. 19**) show a 4 fold decrease in virus quantity with the high IFN-β and high ribavirin treatment. High dose IFN-β and low dose ribavirin reduced the viral titre with almost a 3 fold, while the low dose IFN-β and high dose ribavirin caused a 3.5 fold decrease. The FFA shows a 2.5 fold decrease in viral titre with the low dose of both compounds.

The results of qRT-PCR (**fig. 20**) show that the high dose IFN-β and high dose ribavirin caused just over a 2.5 fold decrease compared to the positive control, high IFN-β and low ribavirin caused a 2 fold decrease, low IFN-β and high ribavirin caused exactly a 2.5 decrease, while the low dose of both compounds caused almost a 1 fold decrease compared to the positive control.

5.2.2 IFN-β and favipiravir (T-705)

The results of the semi-quantitative analysis (**fig. 18**) of IFN-β and favipiravir treatment show a reduction of number of infected cells from the positive control of 95% to 6% with the high dose IFN-β and high dose favipiravir. High dose IFN-β and low dose favipiravir reduced the number to 26%, while low dose IFN-β and high dose favipiravir reduced it to 41.67%. The low dose IFN-β and low dose favipiravir reduced the number of infected cells to 43.33%.
The results of the FFA analysis can be seen in **fig. 19**. High dose IFN-β and high dose favipiravir caused a 4 fold decrease in viral titre. In the case of FFA analysis, the high dose IFN-β and low dose favipiravir caused only a 2.5 fold decrease, while low dose IFN-β and high dose favipiravir caused a 3 fold decrease in viral titre. The low dose IFN-β and low dose favipiravir caused a 2 fold decrease.
The results of the qRT-PCR can be seen in **fig. 20**. The results show that high dose of both compounds caused a 2.5 fold decrease in virus quantity. High dose IFN-β and low dose favipiravir caused a 2 fold decrease, while low dose IFN-β and high dose favipiravir caused only a 1.5 fold decrease in virus quantity. The low dose of both compounds caused just over a 1 fold decrease.

5.2.3 Ribavirin and favipiravir (T-705)

The results of the semi-quantitative analysis are presented in **fig 18**. The results show that the high dose ribavirin and high dose favipiravir caused a reduction in the number of infected cells from the positive control of 95% to 12%. High dose ribavirin and low dose favipiravir reduced the number to 17%, while low dose ribavirin and high dose favipiravir only reduced it to 42.67%. Low dose of both compounds reduced the number of infected cells to 75%.
The results of the FFA analysis can be seen in **fig. 19**. The results show that high dose of both compounds caused a 2 fold decrease of viral titre. According to FFA both high dose ribavirin and low dose favipiravir, and low dose ribavirin and high dose favipiravir caused a 1.5 fold decrease. Low dose of both compounds had nearly no effect of viral titre. The results of the qRT-PCR can be seen in **fig. 20**. High dose of both compounds caused a 2 fold decrease in virus quantity. According to the qRT-PCR high dose ribavirin and low dose favipiravir caused a 1.5 fold decrease, while low dose ribavirin and high dose favipiravir caused less than a 1 fold decrease. Low dose of both compounds had almost no effect on virus quantity.

6 Discussion

The qRT-PCR results for all compounds and combinations generally indicate a higher viral titre. This is because qRT-PCR detects viral RNA which can from whole virus particles, incomplete virus particles or reproductive intermediate, while semi-quantitative fluorescence analysis and FFA detect only whole functioning (infective) virus particles.

6.1 Single compound treatment

IFN-α decreased the viral titres in a dose-dependent manner. The results show that 10 IU/ml reduced viral titres significantly (P=0.0009) compared to the positive control. The results of the FFA and the qRT-PCR are not consistent for the three lower doses of IFN-α. The FFA results indicate that there is antiviral effect with the three lower doses as well, while qRT-PCR results indicate that the effect is minimal. According to qRT-PCR, the virus quantity in the wells treated with the lowest dose was higher than the positive control. This could indicate that 0.01 IU/ml IFN-α has no antiviral effect, and the reason for the higher quantity is that viral propagation is not exactly the same in each well, but on the other hand, PCR detects several versions of viral RNA, and might not represent the quantity of functional virus particles. The effect of 10 IU/ml was significantly greater than the three lower doses (compared to 0.01 IU/ml, where P=0.0026), but there was no significant difference between the three lower doses. The conclusion is that IFN-α has potential antiviral effect with all four concentrations, but a high dose is required to see a significant decrease in viral titres.

All methods indicate that IFN-β has a pronounced antiviral effect that is dose dependent. Statistical comparison of the different doses to the positive control shows that there was significant difference in viral titres with the three higher doses, while no significant difference with the lowest concentration. 10 IU/ml IFN-β significantly reduced the viral titre (P<0,0001). Both FFA and qRT-PCR indicate little or no effect with the lowest dose of IFN-β. There was a highly significant difference between the 10 IU/ml and the lower doses, and the differences between the lower doses were also significant. This indicates that IFN-β has a prominent *in vitro* antiviral effect, also at lower concentrations.

Both IFN-α and IFN-β have noticeable effect on RABV replication in infected N2A cells *in vitro*. IFN-β is significantly better than IFN-α (P=0.0035 at 10 IU/ml), and IFN-β alone had almost the same effect as the combination of the two at all four doses. According to FFA test IFN-β actually had a greater effect alone than in the combination. This indicates that the effect of IFN-β is better than IFN-α, and that in the combination, the effect of IFN-β dominated.

Ribavirin has an antiviral effect that is dose-dependent. 10 µg/ml had a significant effect on the viral titre compared to the positive control (P=0.0022), and the effect was notably greater than in case of the three lower doses. The results indicate a slight antiviral effect with 1 µg/ml. The effect of 0.1 µg/ml is insignificant, and 0.01 µg/ml had no effect. This leads to the conclusion that ribavirin has an effect on RABV replication, but a high dose is needed for a reliable inhibitory activity.

Favipiravir has an antiviral effect similar to ribavirin. 10 µg/ml had a significant effect on viral titres compared to the positive control (P=0.0082). There was still detectable antiviral inhibition of viral propagation with 1 µg/ml and 0.1 µg/ml, while there was no antiviral effect on virus replication with 0.01 µg/ml. 10 µg/ml had a significantly greater effect compared to the other doses, while there was no significant difference between the lower doses. This leads to the same conclusion as for ribavirin; that favipiravir has a dose-dependent antiviral effect, but that a high dose is needed for significant effect.

6.2 Combination treatment

All Combinations of IFN-β and ribavirin were able to reduce viral titres at all tested concentrations. The combination of high dose IFN-β and high dose ribavirin had a highly significant effect on the viral titre compared to the positive control (P<0,0001). Reduced dose of IFN-β and maintained high dose of ribavirin had a greater impact on the viral titre compared to reduced dose ribavirin and maintained high dose IFN-β; indicating that ribavirin had a more important role in the combination, but this difference was rather slight. We have already concluded that both IFN-β and ribavirin had antiviral effect. The combination of the two enhanced the antiviral activity, but the results indicate this effect in more additive than synergistic. IFN-β had a slightly greater effect on its own at a very high dose (10 IU/ml), but the combination of the drugs allows for reduction of the doses of each drug and achieves a better antiviral effect than lower doses of IFN-β treatment.

The combination of IFN-β and favipiravir has a similar effect to that of IFN-β combined with ribavirin. All of the combination doses significantly reduced the viral titres. Same as the IFN-β and ribavirin combination, the high dose of both compounds had a very pronounced effect on the viral titre. The results of the semi-quantitative analysis, FFA and qRT-PCR were not consistent. The FFA results indicated that favipiravir had a more important role in the combination, because when reducing the dose of favipiravir, and maintaining high dose of IFN-β, the viral titre was higher than with the opposite. Both the semi-quantitative analysis and qRT-PCR indicated that IFN-β was more important for the antiviral effect. The combination had a significantly better effect with all dose combinations than the effect of favipiravir alone at any dose. The combination was not significantly better than IFN-β alone at the highest concentration, but like to ribavirin – the combination of lower doses had greater effect on viral replication than lower doses of single IFN-β treatment.

In the case of ribavirin and favipiravir combination, the results of the three methods analysing the effect of the two were not consistent. FFA indicated that the two compounds play an equal role in the antiviral effect of the combination, while the semi-quantitative analysis and qRT-PCR suggest that decreasing the dose of ribavirin reduced the effect more than decreasing the dose of favipiravir. We have previously concluded that both ribavirin and favipiravir had a significant effect on viral replication, but the combination of the two compounds did not significantly enhance the effect.

There was no significant difference between the effect of the combination of IFN-β and ribavirin, and the combination of IFN-β and favipiravir. Nor was significant difference observed between the effect IFN-β combined with favipiravir and the combination of ribavirin and favipiravir. However, the combination of IFN-β and ribavirin inhibited viral multiplication significantly more than the combination of ribavirin and favipiravir (P=0.0006). These results indicate that IFN-β has the most important role in these combinations, secondly ribavirin, and lastly favipiravir.

6.3 Conclusion

In conclusion it can be pronounced that all the substances had a dose-dependent antiviral effect. IFN-β was more effective than IFN-α, and the combination of the two did not significantly enhance the inhibition of RABV replication. Ribavirin and favipiravir are drugs with already established antiviral effect against several viruses. The results of this study show that both compounds had an effect of the multiplication of RABV in N2A cells *in vitro*. Ribavirin was slightly better than favipiravir, and the combination of the two did not significantly enhance the effect. The combination of ribavirin or favipiravir with IFN-β provide greater antiviral activity compared to the use of compounds alone in the same concentration as in the combination, however, the highest IFN-β dose studied (10 IU/ml) was even more effective. Of all the compounds investigated in this study, the results indicate that it is exogenous IFN-β that is responsible for the greatest effect on viral replication; both used alone, and in combinations.

Although these results are promising in terms of interfering with the multiplication of RABV in neuronal cells, it should be taken into consideration that based on *in vitro* results we cannot reliably predict the effect of these compounds when used *in vivo*. Previous attempts to treat clinical rabies with interferons and ribavirin failed (Appolináriol et al. 2013; Fooks et al, 2014) but according to data of this study the underlying cause of this failure was not the inadequacy of these drugs to limit RABV multiplication in neuronal tissue but other factors, which can include suboptimal application and drug formulation, and other pharmacokinetic factors like blood-brain-barrier penetration problems. Therefore, extensive in vivo experiments on animal models (preferably mice) should follow this study to develop a possibly successful protocol that can slow down the progress of the disease. More

compounds, including different anti-rabies monoclonal antibodies could be involved in the research.

In this study we also proved the advantages of a combination approach: combining compounds that are effective even alone can enhance the inhibitory potential of them. The dose of these drugs can be lower when given in combination, which is another important factor. Treating other viral diseases like hepatitis C and AIDS also proved to be more effective with the simultaneous use of different drugs, combinations. Finding new promising combinations for treatment of rabies via *in vitro* studies like this and subsequent *in vivo* experiments can provide valuable data for developing an effective future treatment protocol.

7 Summary

Rabies is a disease caused by a negative-sense single stranded RNA virus. The virus is neurotropic, and causes progressive encephalitis, that invariably leads to death.

There is no effective treatment for clinical rabies; it remains a serious threat to animal and public health in Asia and Africa and causes more than 55 000 human deaths every year. Therefore, finding possible treatment methods is extremely desirable.

During rabies infection type-1 interferons are produced, which leads to the activation of signalling pathways and to the upregulation of interferon-stimulated genes with antiviral activity. Ribavirin and favipiravir are antiviral drugs with known effect against several RNA viruses.

The aim of this study was to investigate the effect of exogenous type 1 interferon (IFN-α/β) treatment on viral replication in RABV-infected mouse neuroblastoma cells *in vitro*, and the possible antiviral effect of ribavirin and favipiravir. First, the infected cell cultures were treated with four different doses (based on antecedent toxicity tests) of the compounds. Subsequently, the infected cells were treated with combinations of the same compound at four different concentrations. The effects were determined based on the reduction in viral replication and so viral quantity and were assessed by semi-quantitative analysis of fluorescence, FFA and qRT-PCR tests.

All of the substances caused significant decrease in viral replication, in a dose-dependent manner. The most pronounced effect was seen in case of IFN-β; which had a highly significant effect on viral multiplication, both alone and in combination with the other compounds investigated. Both ribavirin and favipiravir had antiviral effect on RABV – ribavirin being slightly better than favipiravir. The combination of the substances slightly increased the antiviral effect compared to single compound treatment; however, the effect seemed to be more additive than synergistic.

Concluding the results, it can be pronounced that IFN-β showed the most promising effect, while ribavirin and favipiravir was also able to interfere with viral replication; but further studies are required to evaluate their effect against RABV infection *in vivo*.

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