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Evaluation of *in vitro* inhibitory potential of type-I interferons and different antiviral compounds on rabies virus replication

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ABSTRACT

Five different compounds were tested for their *in vitro* inhibitory effect against RABV multiplication in mouse neuroblastoma (N2A) cell line. N2A cells were infected with the fixed RABV strain CVS-11 one hour prior to adding antivirals or their respective combinations. The infectious titre of RABV as well as the quantity of viral RNA was determined in the cell culturing medium after 48 h. All five tested compounds (mouse interferon (IFN)-α and -β, ribavirin, favipiravir (T-705) and sorafenib) reduced viral replication in a concentration-dependent manner: IFN-β and sorafenib both provided 73.71% relative inhibition of viral replication in the highest non-cytotoxic concentration, while ribavirin caused 48.07%, IFN-α caused 44.87% and favipiravir caused 35.25% relative inhibition, respectively. When applied in combination, their antiviral activity was not synergistic, but a pronounced inhibition was detected when IFN-β was combined with sorafenib, ribavirin, or favipiravir. The highest antiviral effect was caused by the combination of IFN-β and sorafenib (77.19% relative inhibition). In other combinations there was an antagonistic effect detected in the reduction of viral replication. The results demonstrate that these compounds can be promising candidates for a potential combination treatment of rabies, noting that some combinations are not favourable *in vitro*, which makes thorough *in vivo* studies necessary.

1. Introduction

Rabies virus (RABV) is a neurotropic, single-stranded RNA virus that causes rabies encephalitis, a zoonotic disease, which still pose enormous challenge to animal and public health. In the absence of preventive or post-exposure vaccination the disease is almost invariably fatal: after the onset of clinical signs there is no treatment protocol that could save the life of infected patients, leading to the death of more than 60 000 people every year, which number is probably underestimated [1]. To date, less than 20 survivors of rabies were reported, predominantly with severe neurological sequelae [2–7]. Efforts to establish reliable therapeutic strategies have been unsuccessful yet. The "Milwaukee protocol" based upon the survival of an unvaccinated rabies-infected girl after the induction of coma [8] is not recommended any more after multiple failures [9].

Previous studies have highlighted that the mechanism by which RABV inhibits cellular interferon (IFN)-response plays an important role in viral pathogenicity. Shortly, the phosphoprotein (P) of RABV inhibits the phosphorylation of IFN regulatory factor 3 (IRF-3) [10], the translocation of cytoplasmic STAT-1 into the nucleus [11,12], and the IFN-induced DNA-binding of STAT-1 [13]; whereas the nucleoprotein (N) interferes with the activation of Retinoic Inducible Gene I (RIG-I) [14]. All of these are key elements in downstream IFN-signalling and the production of interferon-inducible proteins. Studies also demonstrated that exogenous type-I IFNs can reduce RABV multiplication *in vitro* [15,16].

Ribavirin and favipiravir (T-705) are antiviral drugs with activity against a wide range of RNA viruses. Ribavirin is in use for 30 years in the therapy of hepatitis C and various viral haemorrhagic fevers. It is among the compounds already used several times in human cases of rabies, and it was an element of the Milwaukee protocol along with ketamine and amantadine [8], but provided limited success even after intrathecal administration [17]. However, in cell cultures it can interfere with RABV replication [18]. Favipiravir is a recently discovered compound and is approved in Japan as an anti-influenza agent. It has been tested against a number of RNA viruses [19–25]. Many of these reports suggest that favipiravir could replace the current role of ribavirin in treatment of viral haemorrhagic diseases because it lacks the toxicity that

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ribavirin is known to have. Favipiravir also showed activity against RABV replication [26,27].

Sorafenib is a multikinase inhibitor compound that intervenes in the Raf/MEK/ERK signalling pathway and is currently approved for treatment of advanced hepatocellular carcinomas [28,29]. Recent studies have demonstrated that apart from inhibiting tumour-induced angiogenesis and tumour growth sorafenib has an antiviral effect reducing the replication of hepatitis C virus [30,31]. Since Raf/MEK/ERK-pathways are involved in the molecular pathogenesis of rabies encephalitis [32] sorafenib can be considered as a promising candidate molecule for a possible anti-rabies effect.

The aim of this study was to evaluate the *in vitro* antiviral potency of type-I interferons, ribavirin, favipiravir and sorafenib against RABV. Besides testing them as single compounds, six different combinations (each consisting of two compounds) were also tested to detect a possibly enhanced anti-rabies activity. This combinatory approach is favourable assuming that a potential treatment protocol for human rabies could only be effective as combination therapy.

2. Materials and methods

2.1. Cell line, virus and compounds

Mouse neuroblastoma (N2A) cell line was kindly provided by the Viroscience Lab of Erasmus Medical Center (Rotterdam, NL). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Walkersville, USA) supplemented with 10% foetal bovine serum (FBS, Biowest, Nuaillé, FR) and antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, USA).

The fixed RABV strain CVS-11 (Challenge Virus Standard) was obtained from the Animal and Plant Health Agency, Weybridge, UK and was propagated in N2A cells. The virus stock was established at a titre of 10^5 $TCID_{50}$/ml.

Recombinant mouse interferon (IFN)-alpha and -beta was purchased from Merck (Darmstadt, D). Ribavirin (Virazole) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). T-705 was purchased from BOC Sciences (New York, USA). Sorafenib (sorafenib-tosylate, Nexavar 200 mg tablets) was purchased from Bayer Pharma AG (Berlin, D).

2.2. Post-infection antiviral assay

Every candidate antiviral compound was diluted to four different, non-cytotoxic concentrations with 10-fold serial dilution. The highest concentration was chosen based on the results of a cytotoxicity assay (Cytotoxicity Detection Kit Plus, Roche, Basel, CH) on N2A cells (measuring extracellular lactate-dehydrogenase activity, which is proportional with cell damage) completed for each compound beforehand. When testing combinations of compounds, the concentrations were chosen according to the results of the experiments involving single compounds, which were carried out earlier. The stock solution of the drugs was diluted in DMEM containing 2% FBS to the desired concentration.

Confluent N2A cell cultures were trypsinized to produce a cell suspension, which 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). After a 24 h incubation time medium was discarded and replaced to 50 μl plain DMEM (without FBS) containing 5000 $TCID_{50}$ of CVS-11 virus (MOI = 0.125). Plates were then incubated for 1 h (37 °C 5% CO_2) for virus adsorption. After that, the inoculum was discarded and the antiviral solutions were added to the appropriate wells. After 48 h incubation the supernatant from each well was collected and stored at −80 °C for the purposes of subsequent studies.

To evaluate the level of viral replication in the presence (or absence) of different antiviral compounds or their combinations, the supernatants were titrated on N2A cells in quadruplicates using fluorescent focus assay (FFA). The titration plates were incubated for 48 h and fixed with 80% acetone for 20 min. After fixation, the cells were stained with fluorescent-labelled conjugate of anti-rabies monoclonal immunoglobulin (Fujirebio Diagnostics, Malvern, USA). 50 μl of conjugate was added to every well, followed by a 30-min-long incubation in dark. Finally, cells were washed twice with PBS and the plates were evaluated using an inverted fluorescent microscope (AxioVert 200M, Zeiss, Oberkochen, D). RABV titres were calculated using the Spearman-Kärber method [33,34] and expressed in 50% tissue culture infectious dose ($TCID_{50}$/ml). The results of cell cultures treated with the compounds or their respective combinations were compared to those left untreated (virus control). Relative inhibition of RABV replication was calculated as $[1 - \log_{10}(\text{infectious titre of treated cell culture})/\log_{10}(\text{infectious titre of virus control})] \times 100$.

2.3. Real-time reverse-transcription PCR

To quantify RABV RNA levels in the supernatant samples collected from the cell cultures of the antiviral assay real-time reverse-transcription PCR (qRT-PCR) was performed. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit, Qiagen, Hilden, D according to the manufacturer's instructions.

The single-tube qRT-PCR test was performed using the Qiagen 1-step RT-PCR kit with the primers and probe previously described [35], according to the following thermal profile: 50 °C for 30 min, then 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s. For amplification an Applied Biosystems StepOne Plus real-time PCR machine was used (Thermo Scientific, Wilmington, USA). Using a 6-point standard curve of 10-fold serially diluted CVS-11 RNA extracted from a virus suspension of known titre, RNA loads expressed in $TCID_{50}$-equivalents were calculated from C_t-values. The extrapolated $TCID_{50}$-equivalent values were used to make comparison with results of FFA tests easier. This method to determine $TCID_{50}$-equivalent values with the use of qRT-PCR has already been applied and described for different viruses in a number of studies [36,37]. Each reaction was carried out in duplicates.

2.4. Data analysis

The results of the FFA tests are presented as mean ± standard error of mean (SEM) of three different experiments. Results of qRT-PCR were calculated from threshold cycle data using a standard curve and presented mean ± SEM. 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).

3. Results

3.1. Anti-rabies effect of type-I interferons

FFA was performed on rabies-infected N2A cell cultures treated with interferons to determine their *in vitro* antiviral potency against RABV. Both type-I interferons, IFN-α and IFN-β reduced viral multiplication in a concentration-dependent manner (Fig. 1A). The most concentrated IFN-α solution (10 IU/ml) caused a 3.5 $\log_{10}$ decrease in CVS-11 titres compared to the untreated

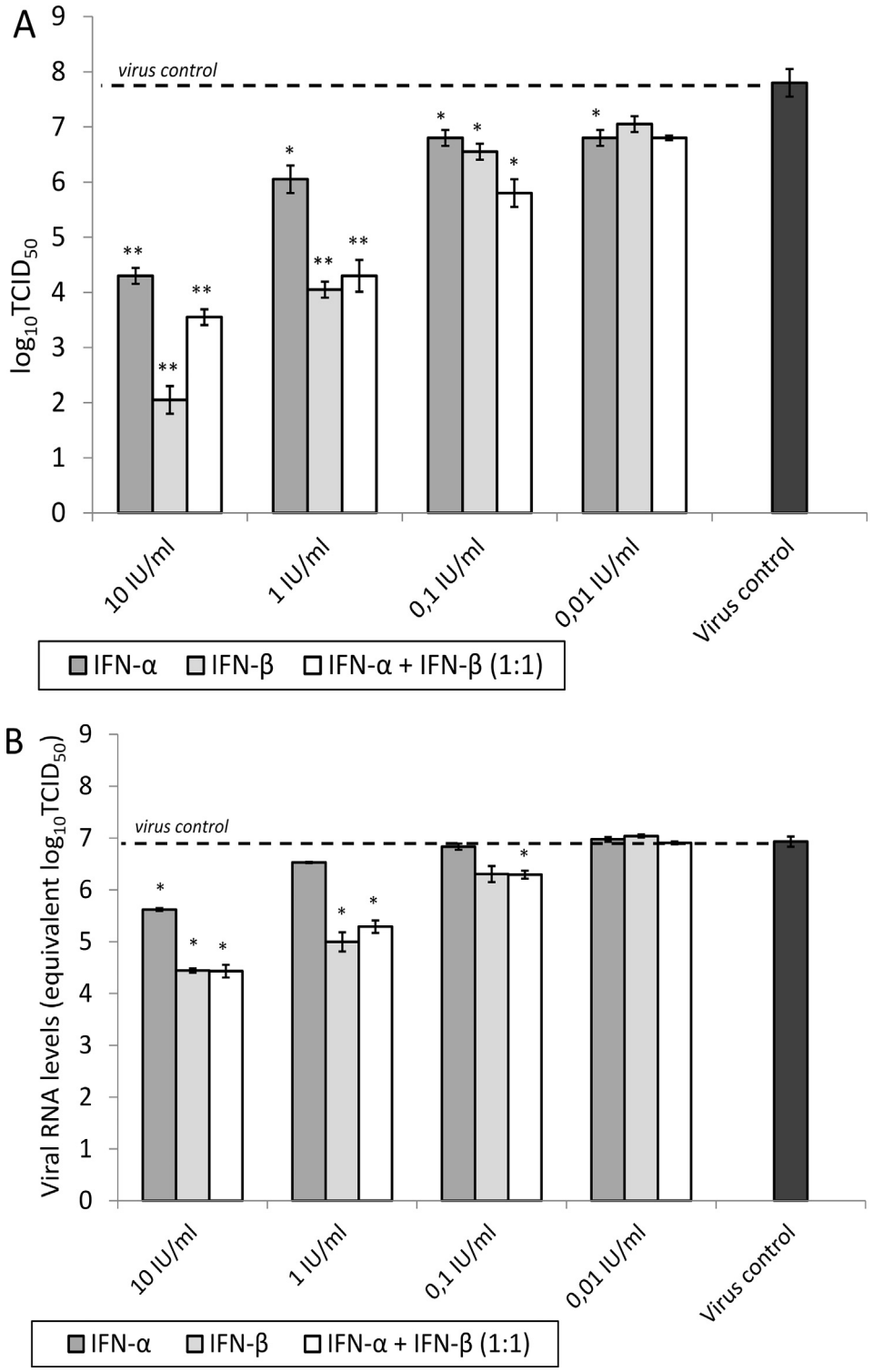

Fig. 1. Anti-rabies effect of type-I interferons in N2A cell culture. (A) viral titres determined with FFA test. (B) viral titre-equivalents calculated from C_t-values determined with qRT-PCR. Values are expressed as mean ± SEM from three independent experiments. Significance levels: * $P < 0.05$; ** $P < 0.001$ compared to the untreated virus control.

virus control, while at 1 IU/ml the decrease was found 1.75 $\log_{10}$. The 0.1 and 0.01 IU/ml caused a slighter but still significant drop in viral titres (1 $\log_{10}$ decrease both cases, $P = 0.0366$). IFN-β revealed to be a more potent inhibitor of RABV multiplication: 5.75 $\log_{10}$ decrease was detected in CVS-11 titres at the highest concentration (10 IU/ml). Titres of RABV in cell cultures treated with 1, 0.1 and 0.01 IU/ml of IFN-β were lower than that of the virus control by 3.75, 1.25 and 0.75 $\log_{10}$, respectively. The difference between IFN-β and -α is significant at the two higher concentrations (inter-group statistical evaluations are presented in Table 2).

The 1:1 combination of IFN-α and -β also reduced RABV multiplication in N2A cells, its effect was stronger than IFN-α but weaker than IFN-β at the two higher concentrations (5–5 IU/ml: 4.25 $\log_{10}$; 0.5–0.5 IU/ml: 3.5 $\log_{10}$ decrease in viral titres; the latter is not significantly different from IFN-β). When applied at a

concentration of 0.05–0.05 IU/ml, titres were 2 $\log_{10}$ lower than in case of the virus control (P = 0.0048), but at the lowest concentration (0.005–0.005 IU/ml) the IFN-mix caused only 1 $\log_{10}$ difference, which is not significant (P = 0.0572) (Fig. 1A). Relative inhibitions of viral replication based on these data are presented in Table 1.

RNA levels in the same supernatant samples were measured using a TaqMan qRT-PCR method and were expressed in $TCID_{50}$-equivalents. Results show the same trends as the FFA test described above: the inhibition of viral multiplication was most pronounced when N2A cells were treated with high concentrations of IFN-β (10 and 1 IU/ml). The corresponding concentrations of the combined IFN-α and -β treatment showed similar efficiency to IFN-β, while the antiviral activity of IFN-α was more moderate and only significant at 10 IU/ml (P = 0.0343) (Fig. 1B).

3.2. Anti-rabies effect of ribavirin and favipiravir

Antiviral drugs ribavirin and favipiravir were used in the same concentrations to treat rabies-infected N2A cells therefore the results for their antiviral assays are discussed together. Both compounds could interfere with viral multiplication *in vitro*. When applied in the lowest concentration (0.01 μg/ml) the reduction of CVS-11 titres was not significant for either of the compounds. At 0.1 μg/ml, favipiravir reduced viral titres by 1 $\log_{10}$ (P = 0.0366), but ribavirin by only 0.75 $\log_{10}$, which is not significant. However, at higher concentrations, ribavirin's effect slightly, but not significantly exceeded that of favipiravir: 3.75 and 2 $\log_{10}$ decrease was caused by ribavirin, while 2.75 and 1.5 $\log_{10}$ caused by favipiravir at 10 and 1 μg/ml, respectively (Fig. 2A). Relative inhibition values are included in Table 1.

CVS-11 titre equivalents calculated from the C_t values of qRT-PCR support the results of FFA test, a concentration-dependent inhibition of viral replication was revealed caused by both compounds, although the differences between treated and untreated cell cultures are lower than in case of FFA (especially in the highest concentration) (Fig. 2B).

Table 1
Relative inhibition of RABV replication caused by different concentrations of antiviral compounds, determined with FFA test. Relative inhibition was calculated as: $[1 - \log_{10}(\text{infectious titre of treated cell culture})/\log_{10}(\text{infectious titre of virus control})] \times 100$.

Compound	Concentration	Relative inhibition
IFN-α	10 IU/ml	44.87%
	1 IU/ml	22.43%
	0.1 IU/ml	12.82%
	0.01 IU/ml	12.82%
IFN-β	10 IU/ml	73.71%
	1 IU/ml	48.07%
	0.1 IU/ml	16.02%
	0.01 IU/ml	9.61%
IFN-α and -β (1:1)	2 × 5 IU/ml	54.48%
	2 × 0.5 IU/ml	44.87%
	2 × 0.05 IU/ml	25.64%
	2 × 0.005 IU/ml	12.82%
Ribavirin	10 μg/ml	48.07%
	1 μg/ml	25.64%
	0.1 μg/ml	9.61%
	0.01 μg/ml	9.61%
Favipiravir	10 μg/ml	35.25%
	1 μg/ml	19.23%
	0.1 μg/ml	12.82%
	0.01 μg/ml	6.41%
Sorafenib	50 μM	73.71%
	5 μM	68.90%
	0.5 μM	16.02%
	0.05 μM	9.61%

3.3. Anti-rabies effect of sorafenib

The anti-RABV activity of the multikinase inhibitor sorafenib was proven to be high, comparable with the effect of IFN-β. Similarly to IFN-β, the highest non-cytotoxic concentration of sorafenib (50 μM) caused a 5.75 $\log_{10}$ drop in CVS-11 titres. When applied at 5 μM, sorafenib was still very effective, resulting in titres 5.37 $\log_{10}$ lower than the virus control. The following concentration (0.5 μM) provided markedly lower inhibition of RABV multiplication: the decrease was only 1.25 $\log_{10}$ (still significant, P = 0.0366), while at 0.05 μM sorafenib failed to interfere with viral replication. Based on these results relative inhibition was calculated for each concentration (Table 1).

Tendencies described above were further confirmed by qRT-PCR, where $TCID_{50}$-equivalent values measured in sorafenib-treated cell cultures where 3.64 and 2.89 $\log_{10}$ lower than that determined in the viral control sample. At 0.5 and 0.05 μM sorafenib concentrations CVS-11 titre equivalents were not significantly lower than in case of the virus control (Fig. 3).

3.4. Anti-rabies effect of antiviral combinations

To reveal whether combinations of the antiviral compounds tested in this study cause an enhanced inhibition of RABV multiplication in N2A cells compared to the effect of the single compounds, 6 different combinations were investigated, each consisting of two compounds. For every compound a higher and a lower concentration was applied, therefore 4 different mixtures were prepared from each combination. In case of type-I interferons, only IFN-β was included in these combinations, because it provided more promising results than IFN-α, and even the combined treatment with the two interferons did not show higher efficiency than IFN-β alone. "High" and "low" concentrations of IFN-β, sorafenib, ribavirin and favipiravir were chosen based on the results of post-infection antiviral assays with single compounds. For IFN-β and sorafenib the "high" concentration (1 IU/ml and 5 μM) was only the second highest in previous experiments, because at the highest doses they reduced RABV titres so effectively that a possible synergistic inhibitory effect of a combination would be difficult to detect. In case of ribavirin and favipiravir the two highest concen-

Table 2
Inter-group statistical evaluations between the antiviral effect (determined with FFA method) of the different compounds at the highest applied concentrations (Student's *t*-test). P values are presented; significant differences (P < 0.05) are highlighted with asterisk.

	IFN-α	IFN-β	IFN-α and -β (1:1)	Ribavirin	Favipiravir	Sorafenib
IFN-α		* 0.0035	* 0.0213	0.5903	* 0.0351	0.1169
IFN-β			* 0.0118	* 0.0164	* 0.0069	1.000
IFN-α and -β (1:1)				0.3213	* 0.0091	0.1825
Ribavirin					0.1201	0.0789
Favipiravir						0.1051
Sorafenib						

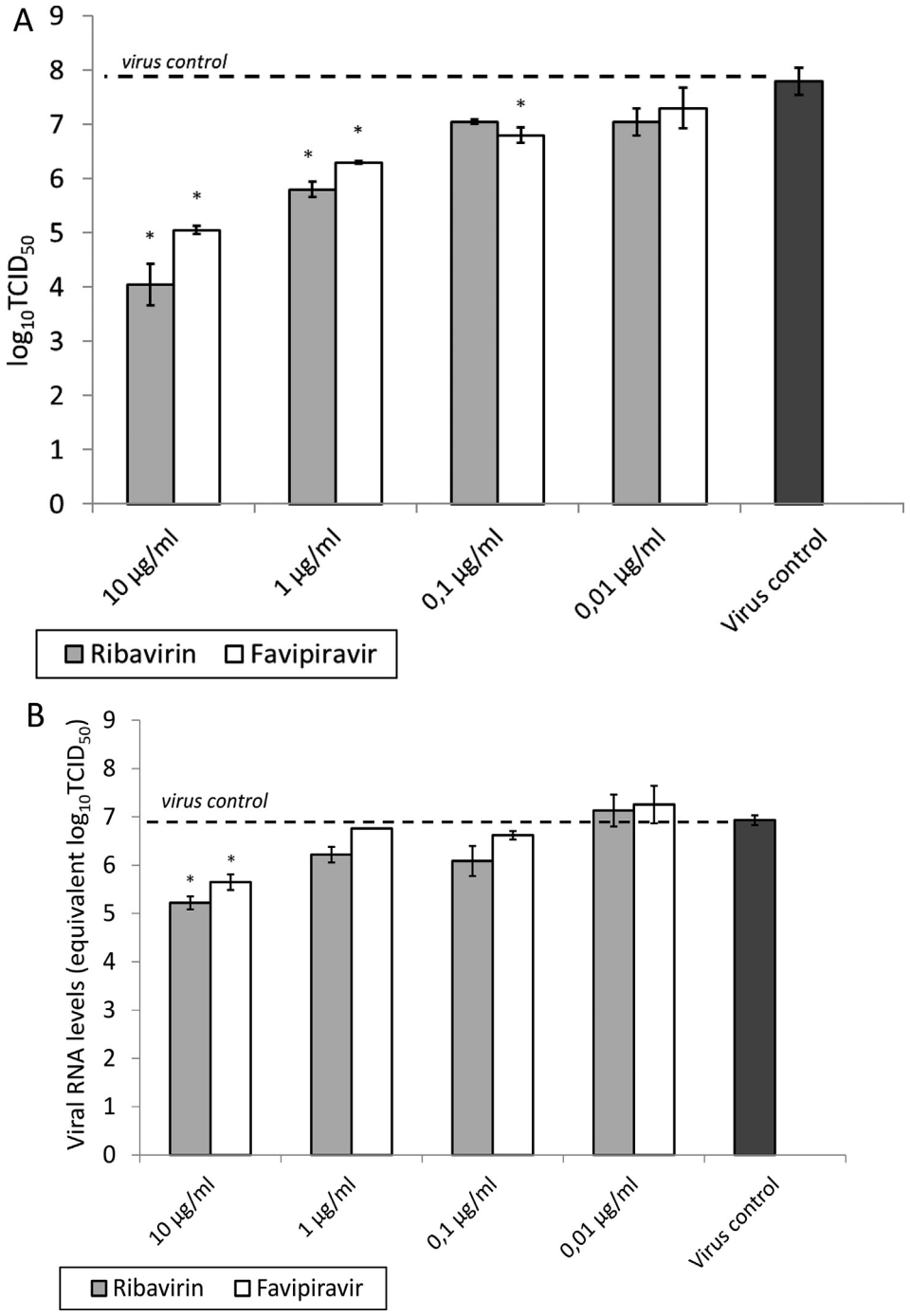

Fig. 2. Anti-rabies effect of ribavirin and favipiravir in N2A cell culture. (A) viral titres determined with FFA test. (B) viral titre-equivalents calculated from C_t-values determined with qRT-PCR. Values are expressed as mean ± SEM from three independent experiments. Significance level: * $P < 0.05$ compared to the untreated virus control.

trations (10 and 1 μg/ml) were involved in the combination study, because lower concentrations failed to significantly interfere with RABV replication.

According to the results of FFA investigations, no synergistic antiviral effect could be detected for any of the applied combinations. The increase in the inhibition of RABV replication, if present, was slight to moderate. It was IFN-β that in combination with any of the three other compounds provided an increase in anti-rabies activity compared to the effect of individual compounds of the combination at the appropriate concentrations (Fig. 5).

From all combinations the mixture of "high" concentrations of IFN-β and sorafenib (1 IU/ml and 5 μM) was the most effective with a relative inhibition exceeding the individual effect of both 5 μM sorafenib and 1 IU/ml IFN-β (77.19% in contrast to 68.90% and 48.07%). When using the lower concentration from either compounds or even from both the combination still improved antiviral activity compared to the effect of same concentrations of IFN-β and sorafenib alone.

When IFN-β was combined with ribavirin and favipiravir the inhibition of RABV replication was also higher compared to the single compounds, except for the combinations of 1 IU/ml IFN-β with 1 μg/ml ribavirin or 1 μg/ml favipiravir with a relative inhibition (40.44% and 36.76%) lower than in case of 1 IU/ml IFN-β alone (48.07%).

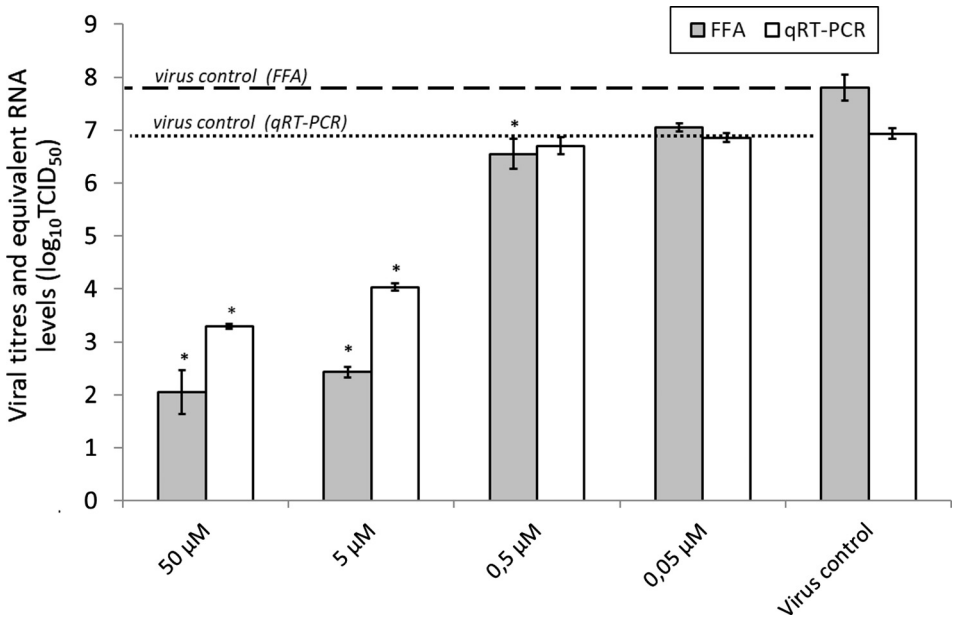

Fig. 3. Anti-rabies effect of sorafenib in N2A cell culture. Values are expressed as mean ± SEM from three independent experiments. Significance level: * $P < 0.05$ compared to the untreated virus control.

All other combinations (sorafenib + ribavirin, sorafenib + favipir avir, ribavirin + favipiravir) reduced RABV multiplication less than the components of the combination alone at the same concentrations.

TaqMan qRT-PCR tests have also been performed for every sample of the combinations. $TCID_{50}$-equivalent titres were calculated from C_t-values. The findings are quite similar to those reported for the experiments with single compounds: at lower RNA levels the extrapolated titre values were considerably higher than the titres determined with FFA, while at higher RNA quantities (equivalent to 10^6 $TCID_{50}$/ml and higher) this difference disappeared (Fig. 4A and B).

4. Discussion

Although preventive and post-exposure vaccination against rabies is successful and well established for many decades, in recent years, research for new options in the therapy of clinical rabies has become more extensive, involving high number of compounds from different origins and mechanism of action [18,26,38–41]. The first step of evaluating the antiviral effect of a candidate molecule is *in vitro* research in cell lines susceptible to the virus in question. This study was performed on N2A mouse neuroblastoma cell line, which is widely used for various *in vitro* assays related to rabies [26,39,42–44].

Type-I IFNs are among the most natural choices when looking for compounds with possible anti-rabies activity. As RABV suppresses the IFN-production in infected host cells by a well-described molecular pathway [10–14], exogenous administration of IFNs can successfully interfere with RABV multiplication in neurons and thus intervene in rabies pathogenesis. Several reports are available that include data for the anti-rabies activity of type-I interferons and interferon-inducible proteins *in vitro* and *in vivo* [15,16,45,46]. Our study not only determined the inhibitory potential of type-I IFNs against RABV replication at four different, non-cytotoxic concentrations, but also compared the effect of IFN-α and -β. In addition, type-I IFNs were used in combinations with the other compounds to reveal a possibly enhanced antiviral activity. Considering subsequent *in vivo* studies on mice, recombinant mouse IFNs were used in the experiments. According to the results, IFN-β provided significant inhibition to RABV in N2A cells in a concentration-dependent manner. IFN-α was found to be less effective, but still significantly reduced RABV titres compared to the untreated control. There was no improvement in antiviral function when the two type-I IFNs were combined compared to the individual use of IFN-β, therefore in subsequent experiments only IFN-β was included in combinations with ribavirin, favipiravir and sorafenib.

Despite being ineffective in human rabies treatment, ribavirin has a confirmed *in vitro* anti-rabies effect in several cell lines [18,47]. Thus, ribavirin was included in this study as a control compound, so the inhibitory potential of other compounds against RABV could be compared to its effect. Furthermore, it was part of the combinations tested in the study, providing more data about its antiviral value. Ribavirin caused a dose-dependent inhibition of RABV replication. At higher concentrations, it was found to be more effective than favipiravir, a novel antiviral compound against RNA viruses. Although in case of certain viruses like the Crimean-Congo haemorrhagic fever virus and the Junin arenavirus favipiravir provides greater inhibition of viral replication than ribavirin [23,25], in our experiments its effect only exceeded ribavirin at lower concentrations. Nevertheless, it still reduced viral titres in a concentration-dependent manner, which is in accordance with the findings of another studies [26,27].

The kinase inhibitor sorafenib was tested based on reports about its activity against the replication of hepatitis C virus [30,31] and the involvement of the MAP-kinase pathway in rabies encephalitis [32]. Hence, previous scientific data only indirectly suggested a possible anti-rabies effect. Surprisingly, it caused an equally great decrease in RABV titres as IFN-β, the most effective antiviral among the other compounds in this study. These data highlight the need for further research on the antiviral potential and detailed mechanism of action of sorafenib and other MAP kinase inhibitors against RABV. In case of other viruses like influenza, there is an increasing interest on the role of MAP kinase pathways in virus infection and replication revealing novel possibilities for antiviral intervention [48–50].

One of the essential goals of this research was to investigate the antiviral activity of combinations of compounds on RABV multipli-

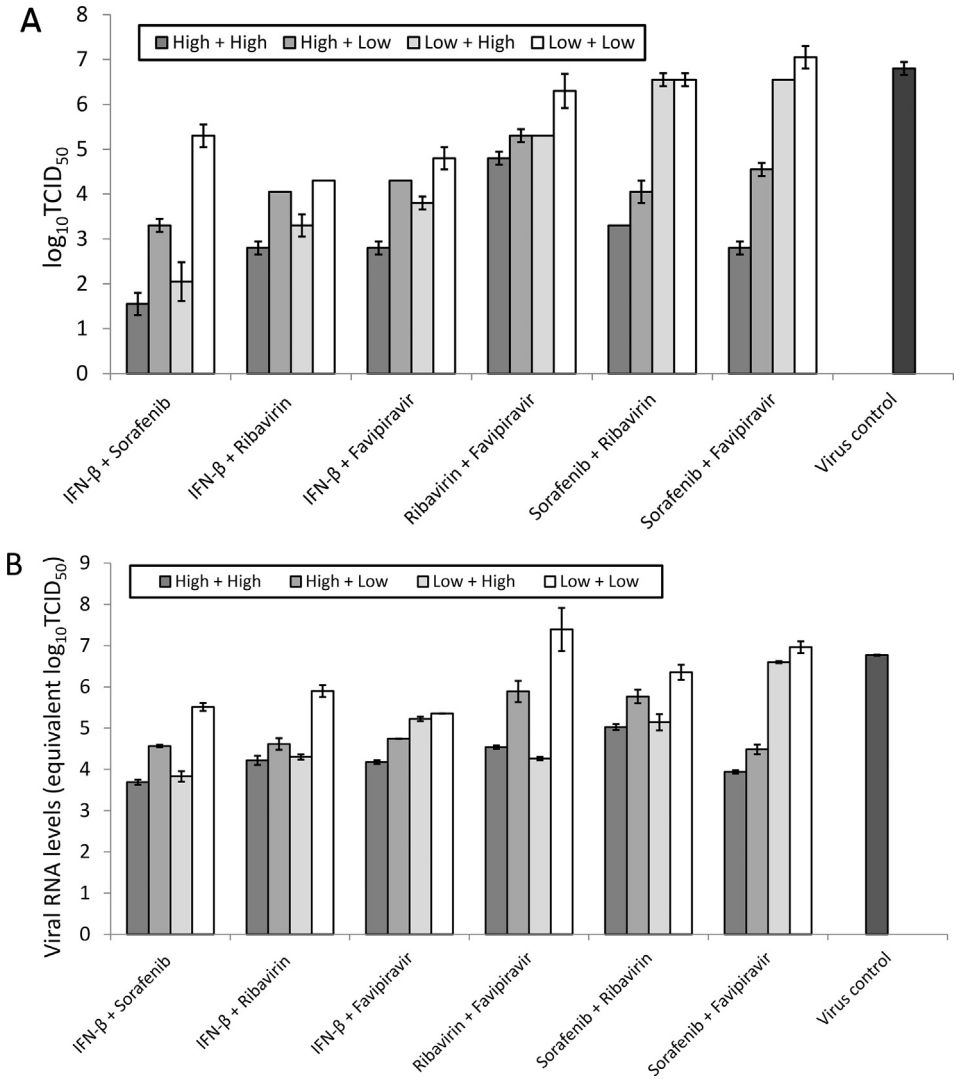

Fig. 4. Anti-rabies effect of combinations of compounds in N2A cell culture. "High" concentrations: IFN-β 1 IU/ml; sorafenib: 5 μM; ribavirin and favipiravir: 10 μg/ml. "Low" concentrations: IFN-β 0.1 IU/ml; sorafenib: 0.5 μM; ribavirin and favipiravir: 1 μg/ml. (A) viral titres determined with FFA test. (B) viral titre-equivalents calculated from C_t-values determined with qRT-PCR. Values are expressed as mean ± SEM from three independent experiments.

cation, since in most studies candidate antiviral molecules are tested only as single compounds. It is unlikely that a possible treatment strategy of human rabies could be successful as monotherapy, therefore *in vitro* data about combinations are valuable for further studies on animal models. When testing combinations of drugs, the greatest scientific impact is achieved if a synergistic effect is detected between the components. In our experiments on rabies-infected N2A cells, none of the combinations showed synergism. In fact, only 3 of the 6 combinations provided an additional inhibitory effect on RABV multiplication compared to the same concentrations of the individual compounds. Among these combinations it was IFN-β combined with sorafenib, which provided the greatest protection against viral replication. This was not unexpected considering that these two compounds were the most effective antivirals when used alone. The greatest reduction of RABV replication in the whole study was achieved when both IFN-β and sorafenib was used at the higher concentration in the combination (1 IU/ml and 5 μM). This combination caused stronger relative inhibition (77.19%) than IFN-β and sorafenib alone even at 10× higher concentrations (10 IU/ml of IFN-β and 50 μM of sorafenib; both providing 73.71% relative inhibition).

The combination of type-I interferons with ribavirin is widely used for the treatment of chronic hepatitis C [51,52]. In case of RABV, we demonstrated that the antiviral effect of IFN-β and ribavirin is slightly increased when they are used in combination, but not in a synergistic manner. Similar results can be reported about the combination of IFN-β with favipiravir where the combined antiviral activity greatly exceeds the individual effect of favipiravir and slightly that of IFN-β. Interestingly, if the concentration of either ribavirin or favipiravir was lowered in combination with the higher dose of IFN-β, the antiviral effect was weaker than in the opposite scenario: lowered concentration of IFN-β combined with the high concentration of ribavirin or favipiravir. This is unexpected, since as a single compound, IFN-β was more effective in reducing RABV titres than ribavirin or favipiravir.

We had high expectations about the combinatory effect of ribavirin and favipiravir as there is an increasing number of studies reporting a pronounced synergy between them leading to a highly enhanced inhibitory effect on various RNA viruses *in vitro* and *in vivo* [23–25]. There is no clear explanation about the background of this synergistic activity. One difficulty in understanding this phenomenon is that the exact mode of action is not elucidated for either ribavirin or favipiravir. Purine analogue function and lethal mutagenesis is among the suggested main mechanisms (ribavirin: [53–55]; favipiravir: [20,21,56]). Our data does not support the hypothesis about a synergistic anti-rabies effect of the

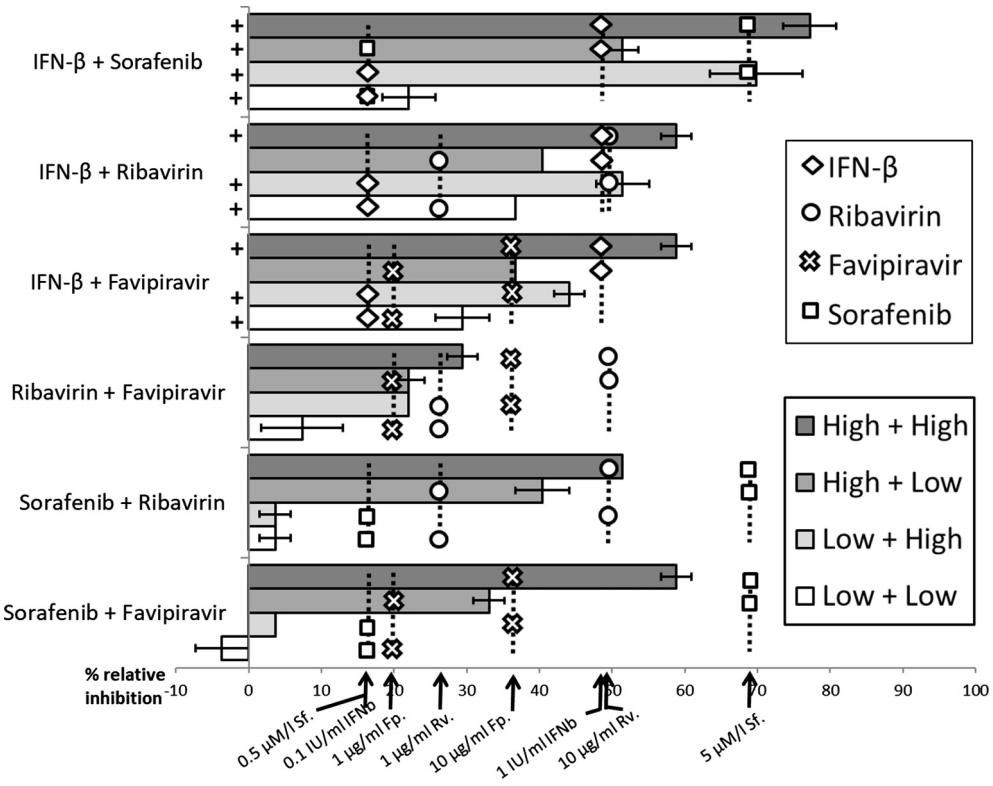

Fig. 5. Relative inhibition of RABV replication caused by the different combinations compared to the effect of individual compounds. Bars: relative inhibition caused by combinations. White indicators: relative inhibition caused by the components of the combinations alone at the same concentration that was used in the combination. "High" concentrations: IFN-β 1 IU/ml; sorafenib: 5 μM; ribavirin and favipiravir: 10 μg/ml. "Low" concentrations: IFN-β 0.1 IU/ml; sorafenib: 0.5 μM; ribavirin and favipiravir: 1 μg/ml. Pronounced antiviral effect (+) is present if relative inhibition caused by the particular combination (bar) is higher than the values of both single compounds (two indicators for each bar). Values are expressed as mean ± SEM from three independent experiments.

ribavirin-favipiravir combination. In order to explain the underlying cause of the lack of an enhanced antiviral activity against rabies, which is present against other viruses, further research is needed.

The combinations of sorafenib with ribavirin or favipiravir were disappointing in terms of reduction of viral replication. The combinations containing sorafenib in the higher concentration (5 μM) reduced RABV titres significantly but less than the same concentration of sorafenib alone. When the concentration of sorafenib was lowered, there was hardly any detectable antiviral effect, even at high concentrations of ribavirin and favipiravir. This shows that there is a clear antagonistic effect between these compounds.

The main conclusions discussed above were made based on results of FFA tests. Apart from this, a TaqMan qRT-PCR assay was also performed for all samples, and viral titre equivalents were calculated from C_t-values. As expected, at higher concentrations of the compounds, which have more pronounced the antiviral effect, extrapolated $TCID_{50}$-equivalents were notably higher than $TCID_{50}$-values found by the FFA test for the same samples. In case of virus titration-based methods like FFA test only complete, functional virions can be detected, whereas in case of RABV PCR all virus-related nucleic acids (including mRNAs, reproductive intermediers and RNAs from incomplete virions) are amplified in the sample. In the current study involving different antiviral compounds with various mechanisms of action some of the compounds and interferon-stimulated genes exert their inhibitory effect on later phases of RABV replication. This leads to the release of a highly reduced number of new infective virions, whereas the amount of virus-specific RNA in the cells does not decrease to such an extent. However, at higher virus loads (around 10^6 $TCID_{50}$/ml and above) this difference is not present, and in some of the samples, FFA-determined titres even exceed those calculated from C_t-values of PCR, probably because the efficiency of amplification is suboptimal at very high RNA load. Taken these differences into account the results gained with two methods are in accordance, showing the same trends, thus PCR results further confirm the findings of FFA.

In conclusion, we demonstrated that the kinase inhibitor sorafenib successfully inhibit RABV replication *in vitro*, leading to a large-scale reduction of viral titres. Besides that, the already reported anti-rabies effect of type-I interferons, ribavirin and favipiravir was confirmed. The combination of IFN-β with any of the other antivirals provided a more pronounced anti-rabies effect compared to the individual compounds. The highest antiviral activity was observed when IFN-β was combined with sorafenib. These results suggest that the compounds involved in this study are promising candidates for a possible combination therapy of human rabies. However, their *in vivo* efficiency and tolerability should be proved in animal models. Furthermore, there is a great need of involving more antiviral compounds into research on rabies therapy or post-exposure prophylaxis, and the evaluation of the effect of combinations is also recommended.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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