Vaccine 37 (2019) 4701–4709

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The effect of combined drugs therapy on the course of clinical rabies infection in a murine model

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ARTICLE INFO

Article history:
Available online 9 April 2018

Keywords:
Clinical rabies
SHBRV-18
Antivirals
Host immune response modulators
Interferon α/β

ABSTRACT

Rabies is a fatal disease of all mammals causing almost 60,000 human deaths every year. To date, there is no effective treatment of clinical rabies once the symptoms appear. Here, we describe the promising effect of combination therapy composed of molecules that target replication of the rabies virus (RV) at different stages of life cycle and molecules that inhibit some pathways of the innate host immune response accompanied by a blood-brain barrier opener on the outcome of RV infection. The study reports statistically significant extension of survival of mice treated with the drug cocktail containing T-705, ribavirin, interferon α/β, caspase-1 inhibitor, TNF-α inhibitor, MAPKs inhibitor and HRIG compared to the survival of mice in the virus control group ($p = 0.0312$). Furthermore, the study points to the significant impact of interferon α/β on the survival of RV-infected mice. We have shown a significant down regulation of pro-inflammatory molecules (caspase-1 and TNF-a) in the CNS in RV-infected mice treated with a combination of drugs including interferon α/β.

1. Introduction

Rabies is an acute, progressive encephalitis caused by neurotropic viruses distributed worldwide. Rabies/the disease is caused by as many as 14 species of RNA viruses within family *Rhabdoviridae* and the genus *Lyssavirus*. All mammals, including humans are susceptible to rabies virus (RV; the prototype lyssavirus) infection. The most important reservoirs are domestic and wildlife carnivores (dogs, foxes) as well as bats. After exposure lyssavirus spread from the site of infection along the peripheral nerves to the central nervous system (CNS) where rapid multiplication occurs. The virus is spread centrifugally via sensory nerves from the brain to different organs, including salivary glands, and it is excreted with saliva.

For centuries various methods of rabies control have been applied. However, a milestone was achieved in the nineteenth century when Pasteur introduced the vaccination against rabies. Rabies vaccine is still the basis for post-exposure prophylaxis (PEP) in combination with hyperimmune rabies antiserum – HRIG – as an interim protection until the host immunity is developed. PEP is effective when applied at the appropriate (very short) time post exposure, suggesting that it prevents the spread of RV to CNS [1,2]. However, delay in PEP administration, inadequate administration or poor vaccine quality leads to the development of clinical rabies. Death occurs within a few days to several weeks after the onset of rabies symptoms. Very few cases of survival of humans with clinical symptoms of rabies were reported so far [3]. There was some hope for effective rabies therapy with an application of the Milwaukee protocol [4], that increased the expectation of therapy of rabies but to date, there is no effective clinical treatment of rabies once the symptoms appear.

A few antiviral compounds have been applied for rabies treatment till now. Ribavirin is an antiviral agent blocking RV replication *in vitro* however, its activity in animals and humans is limited [5]. Studies with interferons in RV infection have indicated their protective role in different animal models. Interferon α (IFN-α) shows some antiviral activity when tested on monkeys challenged with the street rabies virus but the treatment with high doses of the interferon alone or in combination with tribavirin have failed to extend the survival of patients with early clinical rabies encephalitis [6,7]. IFN did not prevent the onset of rabies but delayed the onset of death what suggests IFN as a potential candidate for the treatment of RV infection [8]. Favipiravir

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https://doi.org/10.1016/j.vaccine.2018.04.003
0264-410X/

(T-705) was used *in vitro* and *in vivo* for treatment of many RNA virus infections such as influenza, West Nile, yellow fever, arenaviruses and hantaviruses [9,10]. Recent reports suggest that Favipiravir is an active agent against RV in mice and it is an alternative to HRIG [11]. T-705 alone has reduced morbidity and mortality of mice infected with RV but has not prevented lethality [12].

The main goal of attempts to rabies therapy is not only to stop RV replication but also to limit the virus spread within the CNS and to limit the detrimental host response to RV infection. As it was described previously the application of tumor necrosis factor *a* (TNF-*a)* and mitogen activated protein kinases (MAPKs) inhibitors significantly extended survival of mice infected with RV [13,14]. The use of combination therapy that will operate at different stages of the rabies virus life cycle and targeting simultaneously some pathways of the innate host immune response may be a gateway to the successful treatment of clinical rabies.

One of the problems to overcome in rabies treatment is ineffective drug delivery from the blood to the CNS. The brain is protected by a special barrier (blood brain barrier – BBB) against the influence of different external harmful agents. Unfortunately, the same mechanisms that protect it against external agents also prevent molecule transport from the blood to the CNS. Many existing pharmaceuticals are ineffective in the treatment of cerebral diseases due to the ineffective delivery of the molecules/drugs to the brain. To treat effectively CNS viral infections the active molecules of the drugs must be able to pass the BBB when administered in different routes. In various strategies of the delivery of medical substances to the CNS mannitol was used as a BBB disruption agent. It causes transient/reversible shrinkage of cerebrovascular endothelial cells and permits the therapeutic molecules/compounds to enter CNS that under normal circumstances would not pass the BBB [15].

The aim of this study was to investigate the effect of combination therapy on the outcome of RV infection. Combination therapy composed of molecules that target viral replication at different stages of the rabies virus life cycle and molecules that inhibit some pathways of the innate host immune response accompanied by mannitol as a BBB opener facilitating the transfer of drugs to the CNS. Drugs have been chosen based on the results from previous parts of the project [12,13,21].

2. Materials and methods

2.1. Virus

The Silver Haired Bat Rabies Virus-18 (SHBRV-18) was originally a kind gift from Dr. B. Dietzhold, Jefferson University, USA and obtained from EMC Rotterdam, as a viral stock propagated on murine neuroblastoma N2a cells. Virus titer was determined by end-point titration on BHK-21 cells and calculated using the Spearman – Karber method [16].

2.2. Drugs

A combination of six different compounds selected based on the results of previous studies [12,13,21] was used to formulate the treatment regimen (Table 1). The cocktail consisted: caspase-1 inhibitor (0.2 ng/mouse of Ac-YVAD-cmk from Sigma), TNF-α inhibitor (0.1 mg/mouse of REMICADE from Janssen Pharmaceuticals), MAP-kinase inhibitor (SORAFENIB tosylate 0.6 mg/mouse from Bayer Health Care Pharmaceuticas), mouse IFN-α/β (6000 IU each/mouse from PBL Assay Science), Favipiravir (T705, 6 mg/mouse from BOC Sciences), Ribavirin (3.2 mg/mouse from Tocris Bioscience) and human rabies immunoglobulins (HRIG 0.8 IU/mouse from NIBSC: WHO 2nd International Standard).

Table 1
Overview of experimental groups of mice infected and treated with the drugs combination (ex. 9/0/4-11 ~ a/b/c, a – number of mice in group, b – day of virus infection, c – days of treatment, x – no virus infection).

TreatmentDrug (dose/mouse)	V + D Infected and treated (Humane end-point)	V + D Infected and treated (50% mortality)	D Drug control (Humane end-point)	D Drug control (50% mortality)	V Virus control (Humane end-point)	V Virus control (50% mortality)	BBBO control
	Number of mice/inoculation day/treatment day						
All drugs (V + D_{all}) *IFNα* (6000 IU) INFβ (6000 IU) *T705* (6 mg) *Ribavirin* (3.2 mg) *CASP-1 inhibitor* (0.2 mg) *TNF-α inhibitor* (0.1 mg) *MAPK-inhibitor* (0.6 mg) *HRIG* (0.8 IU) *Mannitol* (25%)	9/0/4–11	9/0/4–11	4/x/4–11 Inoculated with 50 µl of GMEM	4/x/4–11	9/0/4–11 Treated with 1 ml of PBS	4/0/4–11	4/0/4–11 Inoculated with 50 µl of GMEM Treated with 1 ml of PBS
All drugs withoutIFNα/β (V + $D_{all-INFα/β}$) *T705* (6 mg) *Ribavirin* (3.2 mg) *CASP-1 inhibitor* (0.2 mg) *TNF-α inhibitor* (0.1 mg) *MAPK-inhibitor* (0.6 mg) *HRIG* (0.8 IU) *Mannitol* (25%)	13/0/4–11	13/0/4–11	8/x/4–11 Inoculated with 50 µl of GMEM	8/x/4–11	13/0/4–11 Treated with 1 ml of PBS	8/0/4–11	

Mannitol was used as a blood-brain barrier opener (0.5 ml of 25% mannitol from Sigma per mouse).

2.3. Mice

Six-week old female mice C57BL/6 were purchased from Harlan Laboratories, U.S. and housed under specific pathogen free conditions in a biosafety level 3 containment.

2.4. Ethics statement

The experiment was carried out under the Ethics Committee for Experiments on Animals in Lublin, approval No. 90/2015 and all animals were handled in the strict accordance with the good animal practice.

2.5. Animal infection and treatment

Mice randomly assigned to experimental groups as shown in Table 1 were intramuscularly (i.m.) infected in the left hind leg with 50 μl/mouse of the SHBRV-18 strain ($10^{6.13}$ $TCID_{50}$ /ml) on day 0 of the experiment. On the fourth day post inoculation (p.i.) treatment was initiated with the combination of compounds given intraperitoneally (i.p.) 1 ml/mouse. Thirty minutes after the drugs administration mannitol was given i.p. in the respective groups (Table 1). Treatment was given once daily for 8 consecutive days. Control groups included mice that were inoculated with SHBRV-18 only or received the drugs combination only. Mock infected mice received Glasgow Minimum Essential Medium (GMEM). All mice were weighted every day and disease progression was evaluated by scoring clinical signs as follows: score 0: no signs; score 1: ruffled hair and hunched back; score 2: paralysis of the inoculated leg, spasms; score 3: paralysis in both hind legs, severe spasms, circular movement, tail paralysis. Score 3 was considered the humane end point of the experiment and the mice were euthanized and sacrificed by cervical dislocation when reached score 3. For mice which did not show any signs of disease observation was carried out to the 18th day of the experiment.

In the second set of experiments (50% mortality), mice were euthanized in order to compare the effect of the treatment on a pre-determined time point after infection. We assumed the best time point to compare the effect of treatment is when 50% of the virus control mice had succumbed to rabies infection. In 50% mortality experiment mice were euthanized when 50% + 1 of mice in virus control group reached score 3.

Immediately upon euthanasia of mice brain and spinal cord were collected for virological, molecular and histopathological studies.

2.6. Virus titration

CNS samples collected at the time of euthanasia were weighted and 10% (w/v) suspensions were prepared by homogenizing in GMEM medium (Sigma) containing 10% antibiotics mix (10,000 IU penicilin, 10 mg streptomycin, 25 μg amphotericin B from Sigma Aldrich). The homogenates were centrifuged to remove debris, and supernatant was collected and stored at −80 °C until examination. Virus titre (VT) was determined for 10% suspension of the CNS samples as described previously using BHK-21 cells [16].

2.7. Real time PCR

Total RNA was isolated from 10% of the CNS suspension using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's procedures and quantified using Nanodrop (Thermo Fisher Scientific). Viral RNA was detected using SHBRV-18 specific primers/probe combination as described by [13] with a QuantTect Probe RT-PCR Kit (Qiagen) and QuantStudio6 thermocycler (Life Technologies, Thermo Fisher Scientific). To measure the expression level of several markers of a host response and apoptotic/pyroptotic pathways, mRNA was transcribed into cDNA using oligo dT primer (Invitrogen) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The relative level of transcripts was quantified using commercially available primer-probe combinations of the respective genes: CASP-1, CASP-3, cytochrome C, Mcl-1, Bcl-2, TNF-α and Jnk3 (Applied Biosystem, Thermo Fisher Scientific) and TaqMan® Universal PCR Master Mix (Applied Biosystem, Life Technologies). Transcript numbers of the selected genes were expressed relative to the housekeeping gene GAPDH and calculated using the ΔΔCt method [17].

2.8. Histology and immunohistochemistry

For histopathology and immunohistochemistry half of the brain of each mouse was fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned at 4 μm in sagittal plane. Slides were stained with haematoxylin and eosin (HE) for analysis of histopathological lesions by light microscopy. Immunohistochemical analysis for virus antigen detection was performed as follows: tissue sections were deparaffinised in xylene, re-hydrated in descending concentrations of ethanol and incubated for 10 min in 3% H_2O_2 diluted in methanol to block endogenous peroxidase activity, then submitted to heat induced antigen retrieval by incubation in citrate buffer at pH 6.0 for 20 min. The slides were then incubated with polyclonal rabbit antibody against rabies nucleoprotein (Anti-Rabies Nucleocapsid Conjugate, Biorad, USA) as a primary antibody, then with polyclonal goat anti-rabbit IgG -HRP (P044801, Dako, USA, dillution 1:100) as a secondary antibody. For visualization, 3,3′-diaminobenzidine-4HCl (DAB) chromogen (Dako, USA) was used. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Sections incubated without the primary antibody were used to confirm specificity of the staining.

For histopathological evaluation of inflammation, semi quantitative scoring of inflammatory cells was applied. For evaluation of virus antigen distribution, the brain tissue sections were examined for the presence of cells presenting positive IHC labelling and scored using semi quantitative method. The scoring system for the presence of inflammatory mononuclear cells in the same brain regions was as follows: (0) – 0 cells/HPF, (1) – <5 cells/HPF, (2) – 6–15 cells/HPF, (3) – >15 cells/HPF.

2.9. Statistical analysis

Statistical analysis was done using the R studio package and GraphPad 7 Prism. Results of survival time were analysed using a log – rank test. The weight of animals, the virus titre, N gene copies of SHBRV-18 and mRNA levels of the selected genes were first analysed using the Shapiro – Wilk test, to evaluate if they fit a normal distribution. In the case of normal distribution, results from two groups were compared, using the Mann-Whitney U test, Student's t test or Welch's t test. All tests were performed at a level of significance of α=0.05 with $****$ indicating a p value $\leq$ 0.0001, $***$ a p value $\leq$ 0.001, $**$ a p value $\leq$ 0.01 and $*$ a p value $\leq$ 0.05. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Survival time and the animal body weight

To assess the survival of mice SHBRV-18 infected and subsequently treated with the respective drugs combination (V + D) in comparison to the virus control group (V), the percent of surviving animals was plotted against time (Fig. 1). Statistically significant ($p = 0.0312$) extension of survival was recorded in the group of mice treated with all compounds (V + D_{all}) compared to the virus control group. The treatment with the combination of all drugs (V + D_{all}) extended survival of mice to over 9dpi (227.2 hpi, SEM = 28.07) while the average survival time in the V group was slightly over seven dpi (176hpi, SEM = 3.922). One out of nine mice in group V + D_{all} survived up to 429hpi (18 dpi) without any signs of rabies. The application of the treatment composed of all drugs without IFNα/β (V + $D_{all\text{-}INF\alpha/\beta}$) has not extended survival of mice suggesting that IFNα/β may have significant impact on the survival of clinical rabies. An average survival of mice in the group V + $D_{all\text{-}INF\alpha/\beta}$ was 190.3hpi, SEM = 8.469 (almost 8 dpi), very similar to the survival time of the mice in the V group.

No statistically significant differences of body weight means in mice in groups V + D_{all} and V + $D_{all\text{-}IFN\alpha/\beta}$ were seen in comparison to virus control. The mice in the drug control groups (D) did not show any weight loss, suggesting that treatment only did not have any significant effect on the body weight of the mice (Fig. 2).

To investigate the correlation between survival time and virus load in the brain, we compared viral titers and N gene viral RNA in V + D_{all}, V + $D_{all\text{-}INF\alpha/\beta}$ and V groups. Irrespective of the drugs composition in groups V + D_{all} and V + $D_{all\text{-}INF\alpha/\beta}$ significantly lower virus titers (VT) compared to the V group were recorded ($p = 0.0305$; $p = 0.0442$, respectively). In contrast, no statistical differences were recorded in the amount of SHBRV-18 N gene copies between the experimental groups V + D (V + D_{all} and V + $D_{all\text{-}INF\alpha/\beta}$) compared to the V group irrespective of the CNS part (Fig. 3).

Regarding analysis of an overall virus load in the different parts of the CNS, the highest VT as well as viral RNA level was recorded in the spinal cord and the lowest in the brain, regardless of the experimental group V + D_{all} and V + $D_{all\text{-}INF\alpha/\beta}$ or V group and regardless of time of mice euthanasia (human end-point or 50% mortality). The differences were statistically significant ($p \leq 0.05$) (Fig. 4).

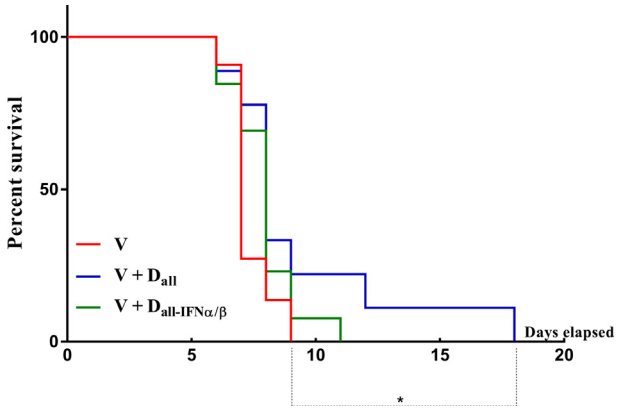

Fig. 1. Percentage survival of mice in experimental groups (red line – virus control, n = 22; blue line – SHBRV-18 infected mice treated with all drugs compound V + D_{all}, n = 9; green line – SHBRV-18 infected mice treated with all drugs compound without IFNαβ,V + $D_{all\text{-}INF\alpha/\beta}$, n = 13). Significant differences were calculated with a log rank test ($\alpha = 0.05$; $^{*}p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

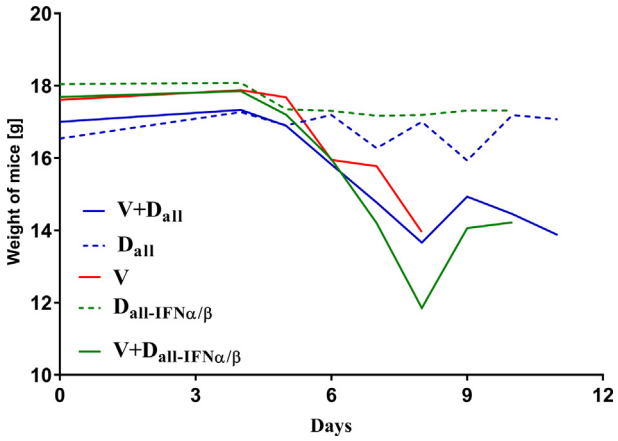

Fig. 2. Weight of SHBRV-18 infected mice in the experimental groups. (red line – virus control (V), n = 22; blue line – mice treated with all drugs compound, (V+D_{all}), n = 9; green line – mice treated with all drugs compound without INFα/β (V+$D_{all\text{-}IFN\alpha/\beta}$); n = 13; blue dotted line – drugs control, D_{all}; n = 4; green dotted line – drugs control, $D_{all\text{-}IFN\alpha/\beta}$; n = 8). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. mRNA gene expression of selected markers

In order to investigate the effect of the compounds that were used to reduce innate host immune response to RV infection a few molecular markers were evaluated. Since our combination treatment included inhibitors of the inflammatory response i.e. cytokine TNF-α and caspase-1 that are involved in induction of an inflammatory form of programmed cell death, (i.e. pyroptosis), the expression of mRNA of relevant markers of pyroptosis (CASP-1), apoptosis (CASP-3, cytochrome C, Mcl-1 and Bcl-2) and proinflammatory markers (TNF-α and Jnk3) were measured (Fig. 5, Table 2).

In the group of mice that were euthanized at humane end points, we found significantly lower mRNA transcripts for TNF-α and CASP-1 in the mice from group V + D_{all} compared to V mice, suggesting an inhibition of inflammatory response in the treated group. However, this was not the case in mice from the group V + $D_{all\text{-}INF\alpha/\beta}$ where the level of mRNA transcripts for TNF-α and CASP-1 was similar to that in the V group. In contrast, the higher level of mRNA for Jnk-3 was demonstrated in the group of mice V + $D_{all\text{-}INF\alpha/\beta}$ compared to the V group (Table 2). In addition, higher levels of mRNA transcripts were measured for the anti-apoptotic markers Mcl-1 and Bcl-2 and for apoptotic marker Casp3 in the V + $D_{all\text{-}INF\alpha/\beta}$ group compared to the V group (Table 2). Cytochrome c was the only apoptotic marker not affected by the treatment.

Next, we investigated mRNA expression of selected markers in the 50% mortality groups. Similarly to the observations in the humane end-point groups, IFNα/β seemed to have an effect on the host inflammatory response as evident by the lower level of mRNA transcripts for TNF-α and Jnk3 in the V + D_{all} group compared to the V group. In addition, Mcl-1 and Bcl-2 markers were also significantly lower in the V + D_{all} group compared to the V group. Significant differences at the levels of selected markers are summarized in Table 3.

In contrast, in the group of mice V + $D_{all\text{-}INF\alpha/\beta}$ with 50% mortality no significant changes in the levels of mRNAs of selected markers were seen in comparison to the V group (Table 3).

3.3. Histopathological analysis and immunohistochemistry

In the histopathological studies the mean scores for the rabies antigen distribution were similar between all mice inoculated with

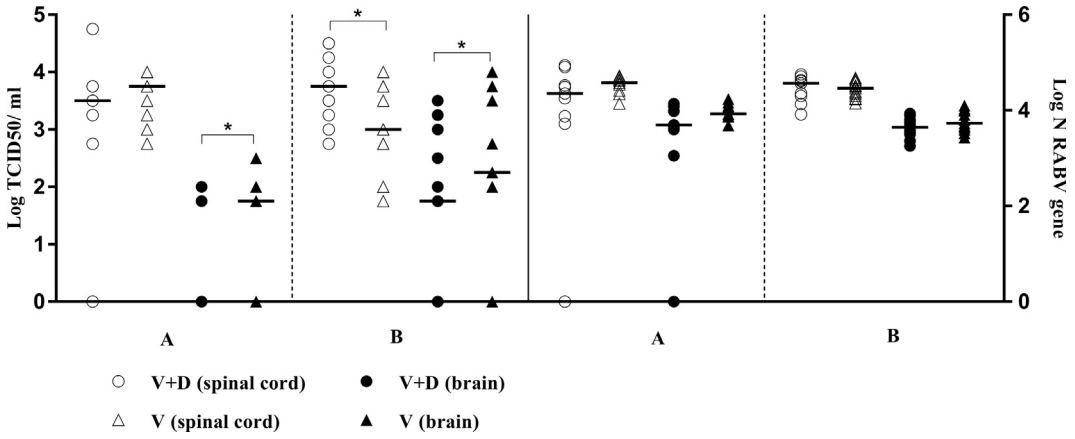

Fig. 3. Virus titer and N gene copies of SHBRV – 18 in different parts of the CNS (white symbols – spinal cord samples; black symbols – brain samples) in two treatment regiments: A – treatment with all drugs, V + D_{all}, B – treatment with all drugs without IFNαβ, V + $D_{all\text{-}IFN\alpha/\beta}$ (white circle – infected and treated, spinal cord, n = 9(A), n = 13(B); white triangle – virus control, V, spinal cord, n = 9(A), n = 13(B); black circle – infected and treated, brain, n = 9(A), n = 13(B); black triangle – virus control, V, brain, n = 9(A), n = 13(B)). Significant differences were calculated using a Mann – Whitney U test, Student's t – test dependent on a normality test ($\alpha = 0.05$; $^{*}p \leq 0.05$). Data is presented as median; n – number of mice.

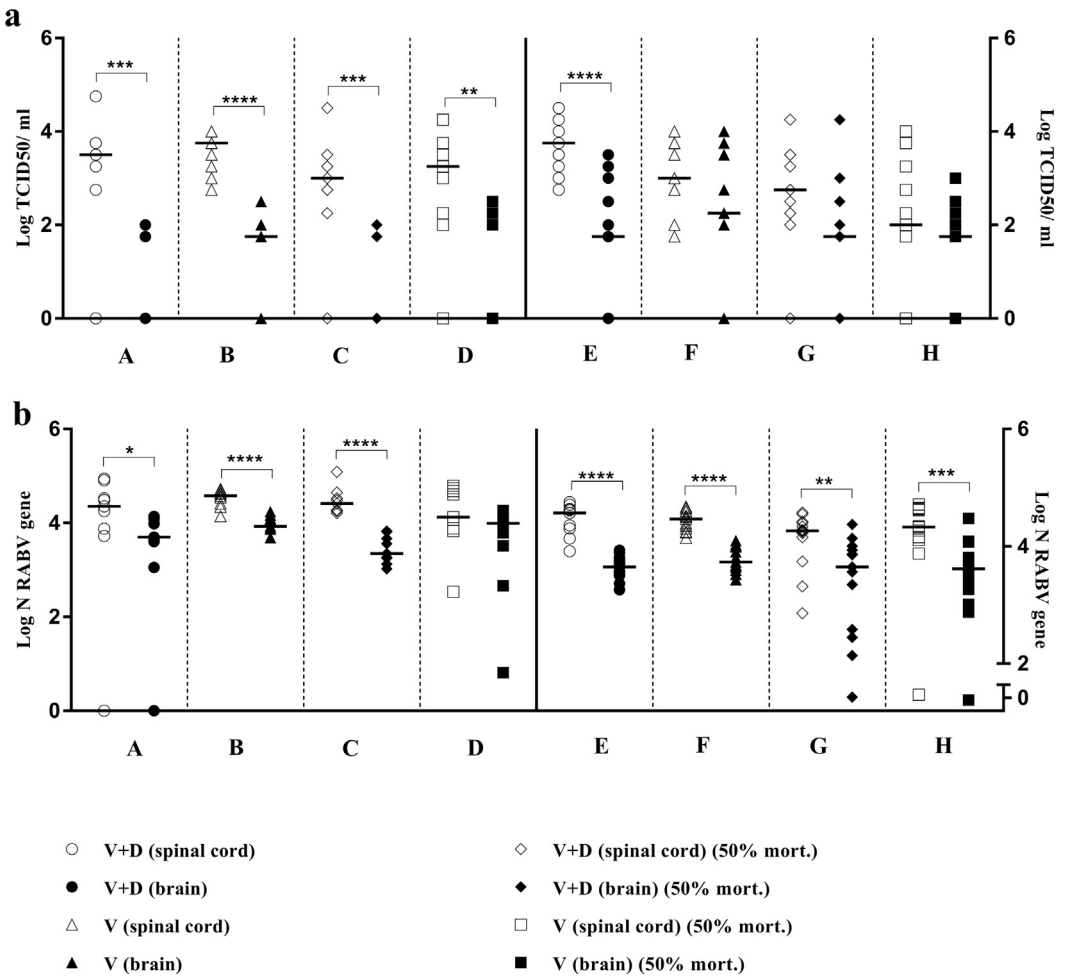

Fig. 4. The comparison of virus titers determined in BHK cells (a) and rabies N gene copies measured by qRT-PCR (b) within experimental groups (A – V + D_{all}, human end-point mortality, n = 9; B – V, human end-point mortality, n = 9; C – V + D_{all}, 50% mortality, n = 9; D – V, 50% mortality, n = 9; E – V + $D_{all\text{-}IFN\alpha/\beta}$, human end-point mortality n = 13, F – V, human end-point mortality, n = 13; G – V + $D_{all\text{-}IFN\alpha/\beta}$, 50% mortality, n = 13; H – V, 50% mortality, n = 13) in different parts of CNS (white symbols – spinal cord samples, black symbols – brain samples). Significant differences were calculated using a Mann – Whitney U test, Student's t – test and Welch's t test dependent on a normality test ($\alpha = 0.05$; $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$). Data are presented as median; n – number of mice.

the virus i.e. V, V + D_{all} and V + $D_{all\text{-}IFN\alpha/\beta}$ groups. There were no differences between the treated groups regardless of mice treated with drugs compound containing INFα/β or not (V + D_{all} and V + $D_{all\text{-}IFN\alpha/\beta}$) (Figs. 6 and 7). Viral antigen was visible in the brainstem, cerebellum, and telencephalon. In the hippocampus no positive reactions for SHBRV-18 antigen was observed except a few

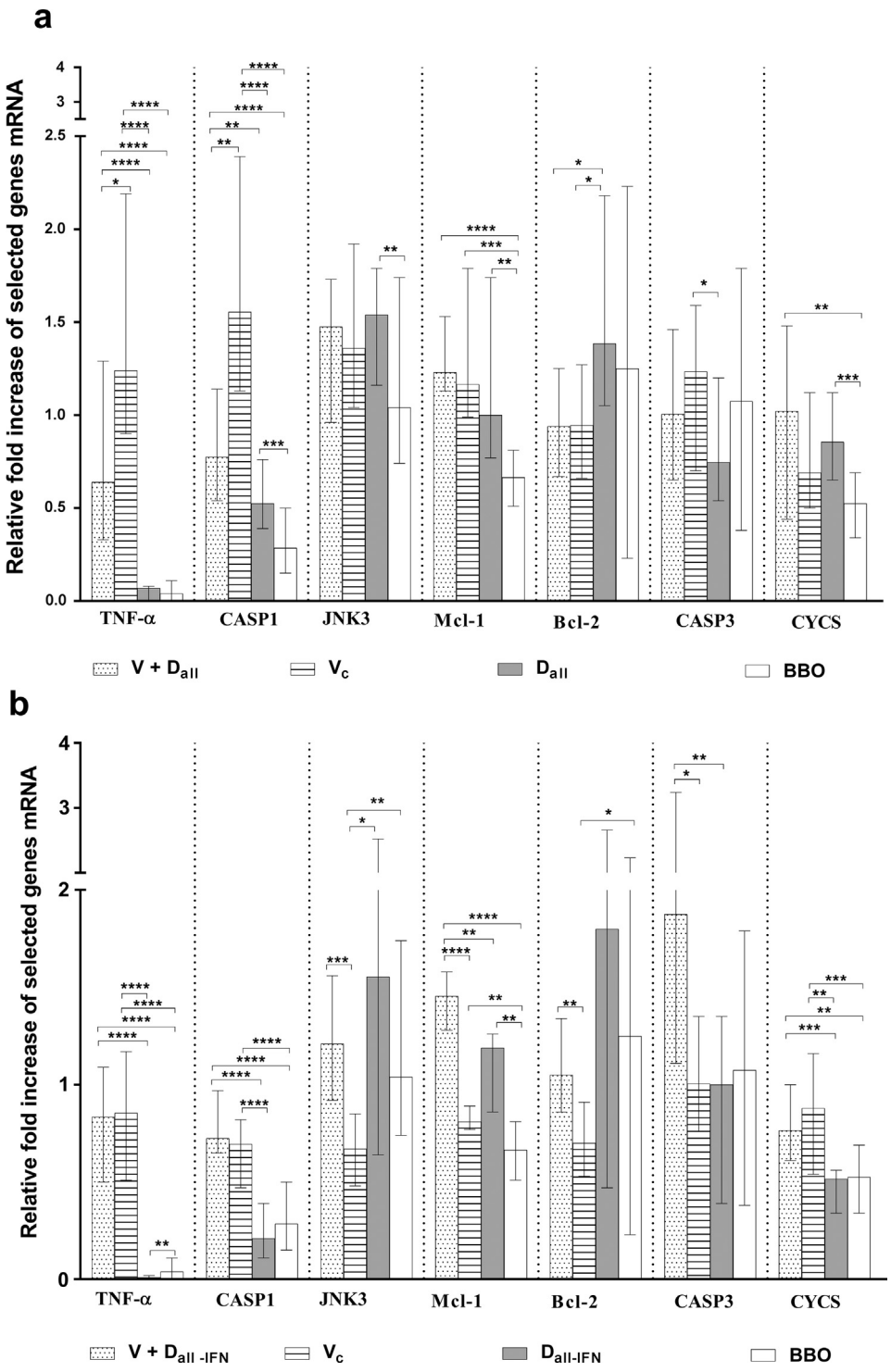

Fig. 5. mRNA levels of selected markers in mice scarified with accordance to human end point scoring. Plot a refers to the treatment regime with all compounds whereas plot b relates to the treatment without IFNα/β (dotted bars – V + D mice, n = 9(a), n = 13(b); hatched bars – V mice, n = 9(a), n = 13(b); grey bars – drug control mice (D), n = 4(a), n = 8(b); white bars – BBBO control, n = 4). Significant differences were calculated using a Mann – Whitney U test, Student's t – test and Welch's t test dependent on a normality test ($\alpha = 0.05$; $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$). Data is presented as median and 95% CI; n – number of mice.

cases in which single positive cells were visible. The immunolabelling was most prominent in the caudal regions of the brain, particularly in the motor related areas of the brainstem: midbrain, pons and medulla, cerebellum and in the somatomotor and somatosensory areas in the isocortex.

In all the groups of mice infected with SHBRV-18 (V, V + D_{all} and V + $D_{all\text{-}INF\alpha/\beta}$ groups) mild to moderate non-suppurative meningoencephalitis was visible, mostly in the caudal regions of the brain, as mild mononuclear cells infiltration of the neuropil, blood vessels and /or sub-meningeal areas. Perivascular cuffing usually associated with gliosis was occasionally observed in the virus control group mainly in the brainstem area (Fig. 8). The differences between the groups of treated mice V + D_{all} and V + $D_{all\text{-}INF\alpha/\beta}$ and the V groups are shown in Fig. 6.

Table 2
Relative change in expression of mRNA for the selected genes in mice sacrificed with accordance to humane end point in the treated groups V + D_{all}; V + $D_{all\text{-}INF\alpha/\beta}$ (↑ – upregulation; ↓ – downregulation) in relation to virus control (V). ns – not significant.

	Humane end-point mortality											
V + D_{all}		TNF - α			CASP1			JNK3			Mcl1	
		V + D	D	BBO	V + D	D	BBO	V + D	D	BBO	V + D	D	BBO
	V	0.0126 ↑	<0.0001 ↑	<0.0001 ↑	0.0017 ↑	<0.0001 ↑	<0.0001 ↑	ns	ns	ns	ns	ns	0.0001 ↑
	V + D		<0.0001 ↑	<0.001 ↑		0.0043 ↑	<0.0001 ↑		ns	ns		ns	<0.0001 ↑
	D	<0.0001 ↑		ns	<0.0001 ↑		0.001 ↑	ns		0.009 ↑	ns		0.0019 ↑
		Bcl-2	CASP3	CYCS									
		V + D	D	BBO	V + D	D	BBO	V + D	D	BBO			
	V	ns	0.0127 ↓	ns	ns	0.0359 ↑	ns	ns	ns	ns			
	V + D		0.0338 ↓	ns		ns	ns		ns	0.0016 ↑			
	D	0.0127 ↓		ns	ns		ns	ns		0.0003 ↑			
V + $D_{all\text{-}INF\alpha/\beta}$													
		TNF - α			CASP1			JNK3			Mcl1		
		V + D	D	BBO	V + D	D	BBO	V + D	D	BBO	V + D	D	BBO
	V	ns	<0.0001 ↑	<0.0001 ↑	ns	<0.0001 ↑	<0.0001 ↑	0.0004 ↓	0.0129 ↓	0.0034 ↓	<0.0001 ↓	ns	0.003 ↑
	V + D		<0.0001 ↑	<0.0001 ↑		<0.0001 ↑	<0.0001 ↑		ns	ns		0.0052 ↑	<0.0001 ↑
	D	<0.0001 ↑		0.0023 ↓	<0.0001 ↑		ns	ns		ns	0.0052 ↑		0.0072 ↑
		Bcl-2	CASP3	CYCS									
		V + D	D	BBO	V + D	D	BBO	V + D	D	BBO			
	V	0.0029 ↓	ns	0.0418 ↓	0.0138 ↓	ns	ns	ns	0.0016 ↑	0.0005 ↑			
	V + D		ns	ns		0.0054 ↑	ns		0.0006 ↑	0.0014 ↑			
	D	ns		ns	0.0054 ↑		ns	0.0006 ↑		ns			

Table 3
Relative change in expression of mRNA for the selected genes in the 50% mortality groups of mice treated V + D_{all}; V + $D_{all\text{-}INF\alpha/\beta}$ (↑ – upregulation; ↓ – downregulation) in relation to virus control (V). ns – not significant.

	50% mortality								
V + D_{all}		TNF – α	CASP1	JNK3	Mcl1				
		V + D	D	V + D	D	V + D	D	V + D	D
	V	0.0308 ↑	<0.0001 ↑	ns	<0.0001 ↑	0.0183 ↑	0.0002 ↓	<0.0001 ↑	<0.0001 ↑
	V + D		<0.0001 ↑		0.0002 ↑		<0.0001 ↓		ns
		Bcl-2	CASP3	CYCS					
		V + D	D	V + D	D	V + D	D		
	V	0.0003 ↑	ns	ns	ns	ns	ns		
	V + D		<0.0001 ↓		ns		ns		
V + $D_{all\text{-}INF\alpha/\beta}$									
		TNF - α	CASP1	JNK3	Mcl1				
		V + D	D	V + D	D	V + D	D	V + D	D
	V	ns	<0.0001 ↑	ns	<0.0001 ↑	ns	0.0008 ↓	ns	0.0081 ↓
	V + D		<0.0001 ↑		<0.0001 ↑		0.0049 ↓		0.0302 ↓
		Bcl-2	CASP3	CYCS					
		V + D	D	V + D	D	V + D	D		
	V	ns	0.032 ↓	ns	ns	ns	ns		
	V + D		0.0189 ↓		ns		ns		

4. Discussion

Earlier experiments with single drugs done under the EU FP7 project No. 602,825 have indicated that Remicade as TNF-α inhibitor, Sorafenib as multi MAPKs inhibitor, Ac-YVAD-cmk as caspase-1 inhibitor extended survival of mice infected with SHBRV-18 [13,21]. Others have demonstrated that the antiviral compounds: T-705, INFα/β and Ribavirin can inhibit RV *in vitro* [8,12]. Mehta el al. [8] have demonstrated that exogenous interferon prolongs survival of rabies infected mice.

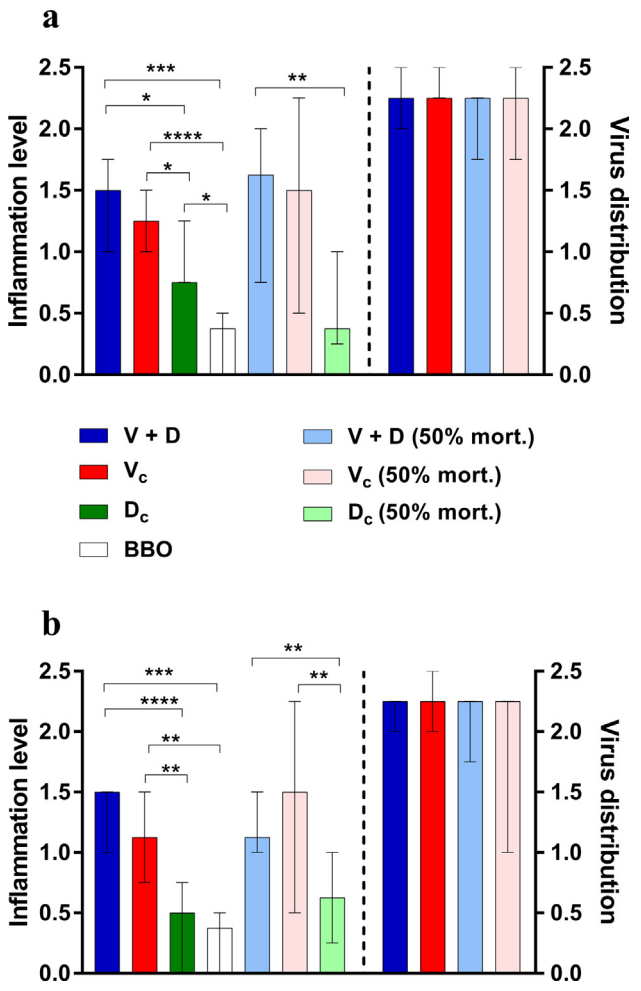

Fig. 6. Inflammation level and virus distribution in the brains of mice treated with all drugs (a) and all drugs without IFNαβ (b). Dark colors refer to mice scarified according to human end point scoring, light colors relate to mice in 50% mortality groups (blue bar – V + D mice, n = 9 (a), n = 13 (b); red bar – virus control V, n = 9 (a), n = 13 (b); green bar – drug control D, n = 4 (a), n = 8 (b); white bar – BBBO control, n = 4). Significant differences were calculated using a Mann – Whitney U test, Student's t – test and Welch's t test dependent on a normality test ($\alpha = 0.05$; $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$). Data is presented as median and 95% CI; n – number of mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

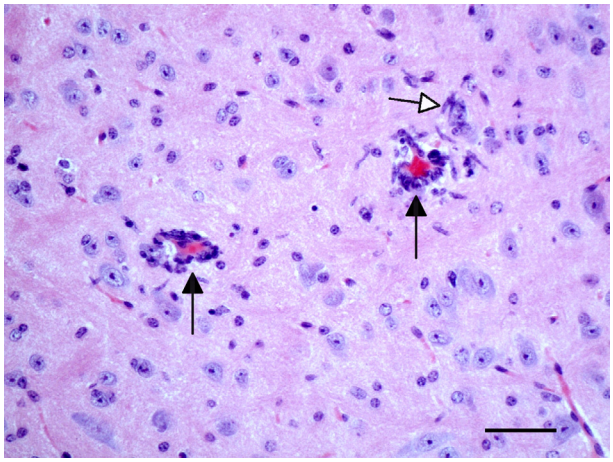

Fig. 7. Mouse, brainstem, virus control group. Histopathological changes representing the inflammation associated with virus infection. Non-suppurative encephalitis characterized by mild perivascular cuffing (black arrows) and activated microglial cells (white arrow). HE. Bar = 50 μm. Presented changes are representative for score 3.

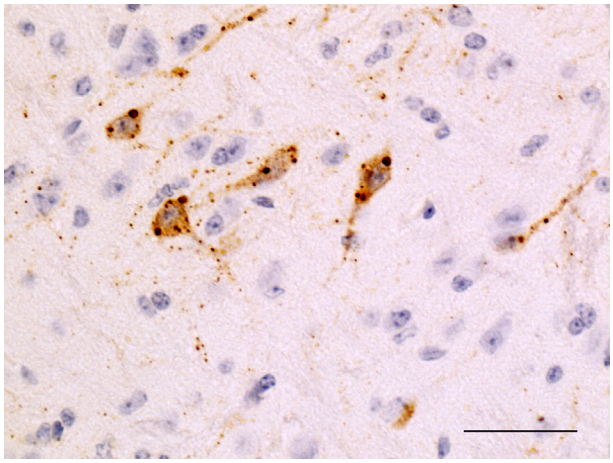

Fig. 8. Mouse, brainstem, virus control group. Immunohistochemical demonstration of virus antigen (brown) in the neurons. IHC, DAB/HRP method. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thus we decided to evaluate the effect of the drugs composition containing of all the compounds implemented with HRIG on the survival of RV infected mice. We hypothesized that a synergistic effect of the drugs applied would result in significant extension of survival time of mice showing rabies signs. Thus the drug cocktail consisted of anti-viral compounds T-705, Ribavirin and INFα/β supplemented with inhibitors of innate host immune response i.e. caspase-1, TNF-α, MAPKs inhibitors and HRIG. The treatment was initiated at 4dpi assuming that this is the time point the virus is most likely present in the CNS but no rabies signs were present yet [18]. In addition, mannitol was administrated acting as a blood brain barrier opener. Generally mannitol disrupts the BBB integrity increasing transiently its permeability enabling the drugs to penetrate the CNS. Thus, the antiviral drugs upon entry into the CNS would block or slow down replication of RV while HRIG would neutralize the virus already present in the CNS and anti-inflammatory drugs would limit damaging innate host immune response within the CNS.

The experiment was done under two treatment regimens: one group of mice was administered with all drugs including INF α/β($V + D_{all}$) and the other group of mice was given all drugs without INF α/β ($V + D_{all\text{-}INF\alpha/\beta}$. The comparison of survival time of the mice in these two regimes group relative to the virus control demonstrated significant extension (almost up to 2 days) of survival of mice in the $V + D_{all}$ group but not in the mice from the $V + D_{all\text{-}INF\alpha/\beta}$. It suggests that the INFα/β has significant impact on the survival of the RV infected mice. These results may also be the effect of almost two fold downregulation of mRNA of proinlammatory cytokines caspase-1 and TNFα recorded in mice from $V + D_{all}$ group relative virus control group. This effect was not observed in the $V + D_{all\text{-}INF\alpha/\beta}$ group (Fig. 5). In addition, the level of mRNA for Jnk-3 protein, that is relatively restricted to the brain and activated in response to the inflammatory cytokines [19], was significantly higher in the mice in the group $V + D_{all\text{-}INF\alpha/\beta}$ relative to the virus control. Mehta et al. has demonstrated that administration of recombinant human interferonα (rhINF-α) significantly prolonged survival of rabies infected mice probably due to the effective decrease of the cytokine expression in the brain [8,20].

To compare the effect of the two different drugs compositions: with and without IFNα/β a common time point of 50%+1 mortality

in the mice in virus control group was applied. At that time all mice in the virus control group were sacrificed and viral and molecular markers were determined. No differences in the virus titer, copy number of virus N gene and mRNA levels of molecular markers attributed to the innate immune host response and apoptosis/pyroptosis of mice treated regardless of the drug composition (with or without INFα/β) and the virus control were recorded. We have used in the experiment a highly pathogenic strain of RV characterized by the rapid development of clinical signs (starting around 5th d.p.i.) thus day 7 when mice were euthanized under the 50% mortality regime might not have been the optimal time point to evaluate the effect of the drug cocktail on the course of RV infection.

Histopatologically we have not observed differences between experimental groups of mice. The virus antigen was the most abundant in the caudal part of the brain particularly in the brainstem and in the somatomotor and somatosensory areas of isocortex in mice irrespective of the experimental group.

In conclusion, our data have shown some beneficial results of using drug combination therapy composed of antiviral and host immune response inhibitors and HRIG as an RV-neutralizing agent and mannitol as a BBB opener. The application of drug cocktail extended mice survival but has not had the fully therapeutic effect. Further studies focusing on optimization of the cocktail composition, initiation of treatment and dose should be considered.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was funded through EU FP7 project ASKLEPIOS – Advanced Studies towards Knowledge on Lyssavirus Encephalitis Pathogenesis Improving Options for Survival (grant 602825). We want to thank all members of the Consortium for the excellent cooperation, fruitful discussions and the members of AB for their support during meetings presented interim reports.

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