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To cite this version:

Sophie Rogée, Florence Larrous, Dirk Jochmans, Youcef Ben-Khalifa, Johan Neyts, et al.. Pyrimethamine inhibits rabies virus replication in vitro. Antiviral Research, 2019, 161, pp.1-9. 10.1016/j.antiviral.2018.10.016 . pasteur-02868865

HAL Id: pasteur-02868865

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Antiviral Research 161 (2019) 1–9

Contents lists available at ScienceDirect

Antiviral Research

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

Pyrimethamine inhibits rabies virus replication *in vitro*

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ARTICLE INFO

Keywords:
Pyrimethamine
Rabies infection
Immune response
Antiviral
Synergy action

ABSTRACT

Rabies virus transmits from animals to humans and causes encephalitis. Every year more than 15 million people receive a post exposure prophylaxis (PEP) treatment that is highly effective in the prevention of rabies disease. However, when clinical symptoms appear, for example in people who did not receive PEP, rabies is almost invariably fatal. Due to the limited access to PEP in some target populations, mostly in Asia and in Africa, rabies causes at least 59,000 deaths a year. PEP is not effective after the onset of symptoms and attempts to develop a treatment for clinical rabies have been unsuccessful. After screening a library of 385 FDA-approved drugs, we found that pyrimethamine inhibits rabies infection *in vitro* through the inhibition of adenosine synthesis. In addition, this compound shows a synergistic interaction with ribavirin. Unfortunately, in rabies infected-mice, pyrimethamine showed no efficacy. One possible explanation may be that the antiviral effect is negated by the observed interference of pyrimethamine with the innate immune response.

1. Introduction

Rabies virus (RABV) belongs to the *Lyssavirus* genus in the *Rhabdoviridae* family and *Mononegavirales* order. Lyssaviruses are transmitted from animals (dogs, cats, bats, etc) to humans by bites, scratches, licking of broken skin and contact of infectious material with the mucosae. They cause encephalitis that is almost invariably fatal in non-flying mammals and in humans (Fooks et al., 2017).

Numerous wildlife mammals, including bats, act as a reservoir, but dog rabies is responsible for 98% of human fatalities (Hampson et al., 2015). Whereas extensive efforts in developed countries have largely controlled dog- (North America, Europe and in a lesser extent South America) and fox- (Western and Central Europe) rabies, the epidemiological situation remains critical in Asia and in Africa (WHO, 2017).

In humans, rabies prevention is achieved by either pre- or post-exposure prophylaxis. In case of exposure to RABV, post-exposure prophylaxis (PEP) is recommended to prevent the spread of the infection and clinical disease. However, current PEP efficacy decreases as the time span between exposure and PEP administration increases, until becoming completely ineffective from the development of symptoms (WHO WER, 2018). Each year, around 19 to 50 million people receive PEP administration, consisting of multiple injections of vaccine together with rabies immunoglobulins (RIG) in the case of high risk exposures (Hampson et al., 2015). Passive immunotherapy is based on human or equine immunoglobulin or F (ab')2 fragments. The availability of this expensive immune serum is limited, and it requires a cold-chain for storage and transport (Bourhy et al., 2009; WHO WER, 2018). Cocktails of human monoclonal antibodies to replace RIG are in development (De Benedictis et al., 2016).

In Europe, the incidence of rabies in humans is low due to the elimination of dog- and fox-rabies. However, continuing surveillance of domestic and wildlife species is necessary due to periodic importation of RABV-infected animals (Ribadeau-Dumas et al., 2016). Unfortunately, Rabies is a neglected and forgotten disease in many countries (Dodet and Africa Rabies Bureau (AfroREB), 2009) and control measures are incompletely implemented for many reasons (Bourhy et al., 2010; Shantavasinkul and Wilde, 2011; Wilde and Lumlertdacha, 2011). Recent estimations of the burden of rabies indicates that around 59,000 humans succumb every year mainly in rural areas of Africa and Asia (Hampson et al., 2015). The life of these patients could be saved if potent inhibitors of rabies virus were available. Therefore, WHO has recognized the need to improve the accessibility and the compliance to PEP (through the development of more effective products and with a low cost) and to develop a rabies treatment in symptomatic patients.

For RABV only limited efforts have been made to discover antivirals (Assenberg et al., 2010; Coutard et al., 2008; Dacheux et al., 2010). In

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https://doi.org/10.1016/j.antiviral.2018.10.016
Received 1 June 2018; Received in revised form 21 October 2018; Accepted 22 October 2018
Available online 25 October 2018
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recent years, a treatment protocol, known as the Milwaukee protocol, was set up that involved induction of coma and treatment with ketamine (a neuroprotective anaesthetic with putative anti-rabies activity (Willoughby et al., 2005). Although both amantadine and ribavirin inhibit the *in vitro* RABV replication (Superti et al., 1985) to some extent, activity has never been demonstrated in experimental infection models or in the clinical setting (Bussereau et al., 1988; Warrell et al., 1989). Despite the fact that the Milwaukee protocol has been used several times, the effectiveness remains controversial (Hemachudha et al., 2006; Jackson, 2012, 2013; Santos et al., 2012). These findings also demonstrate that the application and promotion of a therapeutic protocol that has not been validated with robust and reproducible antiviral studies *in vitro* and in animal models may lead to unproductive, disappointing results.

Recently Favipiravir, a broad spectrum RNA-virus inhibitor, was found to have moderate activity on RABV *in vitro* (EC_{50} ~40 μM) (Yamada et al., 2016). When administered for 7 days, starting 1 h after inoculation, it delayed morbidity and mortality in RABV-infected mice but did not completely prevent lethality. Potent inhibitors of RABV replication that penetrate into the brain are therefore urgently needed.

To identify an inhibitor of RABV, a screening of a library of 385 FDA-approved drugs was performed. If one could find a potent inhibitor in this set of compounds this may speed-up the development of potential applications as many important aspects of these molecules are already known (side-effects, formulation strategies, …). This principle is widely known as drug repurposing. Pyrimethamine was found to inhibit RABV replication *in vitro* at low-micromolar concentrations. In addition, this compound showed a synergistic interaction with ribavirin. Nevertheless, in rabies infected-mice, pyrimethamine showed no efficacy. This can be explained by our observation that pyrimethamine interferes with the innate immune response.

2. Materials and methods

2.1. Cells and viruses

BSR T7/5 cells (Buchholz et al., 1999) and the BHK-T7 cells were grown in Glasgow medium supplemented with 10% calf serum, tryptose phosphate, non-essential amino acids and antibiotics. STING-37 cells were kindly provided by Dr Lucas-Hounari (Institut Pasteur) (Lucas-Hourani et al., 2013). These are HEK293 cells stably transfected by a reporter plasmid carrying the luciferase gene under the control of fifteen interferon-stimulated response elements (ISRE). STING-37 cells, BSR cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were maintained in a humidified incubator with 5% CO2. All cell lines were passaged twice a week.

Several lyssavirus strains were used including a field dog RABV isolate from Thailand, 8743THA (Tha) (Thongcharoen et al., 1990), 9001FRA (FRA), a desmodus type of RABV isolated in French Guyana which belong to RABV species, CVS, a laboratory pathogenic RABV strain, European bat lyssavirus 1 (EBLV-1), 8918FRA and Lagos bat virus (LBV), 8619NIG (Delmas et al., 2008).

2.2. Compounds libraries and reagents

The FDA-approved Drug Library (Catalog No. L1300) was obtained from Selleckchem (Houston, TX) and consisted of 385 compounds at the time of purchase (Table S1).

Pyrimethamine (46706 FLUKA), ribavirin, recombinant human IFN-α, adenosine, guanosine, cytosine and uridine were purchased from Sigma-Aldrich (France, Sigma-Aldrich). All compounds were dissolved in dimethyl sulfoxide (DMSO) while IFN-alpha was dissolved in PBS.

2.3. Antiviral activity

Compound DMSO stocks were diluted 100-fold to a concentration of 66 μM in DMEM containing 1% FCS and 100 μL of these compound dilutions were added to each well of 96-well plates. Then, 50 μL/well of BSR cells at 1.10^6 cells/mL and 50 μL of Tha rabies virus (200 fluorescent forming units, FFU; MOI: 0.0002) were added which brings the final test concentration of the compounds to 33 μM. As a solvent control, a subset of wells was given 0.5% DMSO instead of compound dilution. Non treated-cells infected by Tha virus were used as a positive infection control. At day 2 post-infection (dpi), cells were fixed and the amount of viral nucleoprotein (a major component of viral nucleocapsid present in the cytoplasm) in treated wells relative to wells with only DMSO was measured by ELISA using a mixture of two monoclonal antibodies targeting the rabies nucleoprotein and conjugated to HRP. The absorbance was measured at 450 nm and 600 nm. For each compound, the amount of nucleoprotein was calculated as the ratio of absorbance value (OD 600 nm – OD 450 nm) to the mean of absorbance values obtained from untreated-infected-wells (relative infection 100%). The cytotoxic effects of compound treatment was determined in parallel plates containing mock-infected cells the using ATPlite assay (France, Perkin Elmer) according to the manufacturer's protocol. The antiviral 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) were calculated by nonlinear regression analysis (GraphPad Prism). Combination data analysis was analysed using Horizon ChaliceTM Analyzer Software. Each experiment was performed in triplicate.

2.4. Minigenome assay

The minigenome plasmid pSDI-Tha-CAT (−) was constructed by replacing the sequence of SAD in the pSDI-HH-flash-SC vector (Ghanem et al., 2012) by the extremities of the Tha virus genome, flanking a CAT reporter gene. BHK-T7 cells were transfected in 6-well plates with 1 μg of pSDI-Tha-CAT (−), 10 ng pCMV-RL 1 μg of N-pTIT, 0.5 μg of P-pTIT and 0.5 μg of L-pTIT using 6 μL of lipofectamine 2000 (Invitrogen). In the negative control, one of the pTIT vectors was omitted. Forty-eight hours post transfection, cells were harvested in passive lysis buffer (Roche) and lysates were subjected to reporter CAT assays using the kit « ELISA CAT assay » (France, Roche) according to manufacturer's instruction. Each lysate was tested undiluted and on three serial one to ten dilutions. The renilla activity was controlled with the kit « Renilla-Glo » (Promega) according to manufacturer's instruction on undiluted lysate.

To investigate the inhibitor potency of pyrimethamine, cells were treated with 100 μM of compound starting 24 h post-transfection. The CAT expression was quantified by ELISA CAT assay 48 h post-transfection as described before.

To test the competition with nucleosides, cells were incubated 24 h post-transfection with 25 μg/mL of pyrimethamine or DMSO alone and culture medium was supplemented with 10 μg/mL of nucleoside. At 48 h post-transfection, the CAT expression was measured as described before. Experiments were performed in duplicate.

2.5. In vivo experiment

The protocol of the animal experiment was approved by the French Administration (Ministère de l'Enseignement et de la Recherche) under the number 2013-063 and all experiments were performed in accordance with the relevant guidelines and regulations All animals were handled in strict accordance with good animal practice.

Four week-old Balb/C mice were infected by intra-muscular injection (i.m) with different concentration of Tha virus in 200 μL of DMEM (2 × 50 μL/leg). To investigate the potential protective role of pyrimethamine, mice were separated in 2 groups: one with drinking water (pH = 5) supplemented with 70 μg/mL (281 μM) of compound and 1%

DMSO and the other one with drinking water supplemented with 1% DMSO alone. In these *in vivo* experiments, pyrimethamine was dosed and administered in the same way as in published experiments where it showed successful inhibition of *Plasmodium* replication (Janse et al., 2006; Friesen et al., 2011). The treatment began 4 days before inoculation and continued during 22 dpi. Mice were examined daily for symptoms of rabies (i.e., ataxia, paralysis).

2.6. Activation of ISRE-luciferase reporter gene assay

STING-37 cells that express the luciferase under the control of ISRE were plated at 1×10^6 cells/mL in 96-well plates in 100 µL of medium. Then cells were infected or not with Tha or CVS virus at different multiplicity of infection (MOI, range 0.05–1 FFU/cell) and incubated with increasing doses of pyrimethamine and/or ribavirin or DMSO alone. After 24 h, cells were lysed, and luciferase was quantified using the *Firefly* Luciferase kit (France, Promega).

To determine the role of the drug in innate response, the same experiment was performed but the cells were stimulated with increased dose of IFN-α (I4401 from Sigma).

2.7. Statistical analysis

Data are presented as the mean ± SEM of triplicate determinations and are representative of results obtained in three independent experiments. All analyses were computed with GraphPad Prism software. Statistical significance was assessed using Student's t-tests and defined as $p < 0.05$.

3. Results

3.1. Pyrimethamine inhibits RABV infection

A primary library of 385 FDA-approved drugs was screened for antiviral activity against Tha virus (Table S1) at a concentration of 33 µM. From this compound library only pyrimethamine was identified as a selective inhibitor of RABV infection. A confirmation experiment showed that it inhibits RABV infection for 50% at 3.72 µM (EC_{50}) with a 50% of toxicity (CC_{50}) value of 11.1 µM (Fig. 1). Pyrimethamine is a diaminopyridine derivative that inhibits the dihydrofolate reductase (DHFR) and blocks *de novo* purine synthesis.

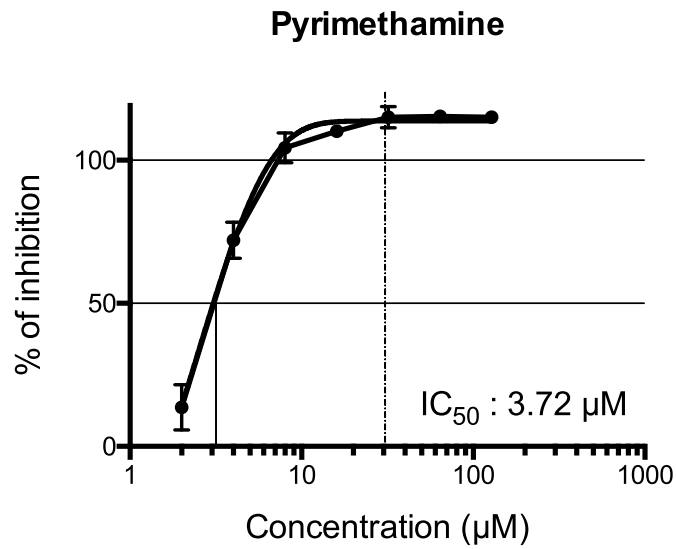

Fig. 1. Dose dependent inhibition of rabies virus replication by pirimethamine. BSR cells were infected with Tha isolate (200 UFF) in the presence of serial dilution of pyrimethamine. At 48 h p.i., using ELISA, percentage of inhibition of infection was calculated as the ratio of absorbance value (OD 600 nm – OD 450 nm) to the mean of absorbance values obtained from untreated-infected-cell (relative infection 100%). In addition, the toxicity of pyrimethamine treatment was monitored in parallel mock-infected cells using ATPlite luminescence assay system. The half maximum inhibitory concentration (EC_{50}) was determined and the threshold of toxicity (50% of toxicity) is indicated by the vertical dash line.

3.2. Synergistic effects of pyrimethamine and ribavirin

In a subsequent experiment we explored the interaction between pyrimethamine and ribavirin, a purine nucleoside analog known to inhibit viral messenger RNA production (Crotty et al., 2000; Tam et al., 2001). Ribavirin by itself showed good efficacy (EC_{50} 7.96 µM) with a CC_{50} value of 72.0 µM (Fig. 2A). Then, a systematic dose-response screen of single and combined compounds at different concentrations was performed and the potential synergistic or additive effects were investigated. The combination of pyrimethamine and ribavirin showed a significant increased antiviral effect in comparison with cells treated with only one compound. Indeed, the EC_{50} value of ribavirin decreased from 7.9 µM to 1.3 µM (with CC_{50} values of 72.0 µM and 72.2 µM, respectively) when combined with 2 µM of pyrimethamine (Fig. 2B). Ribavirin decreased the EC_{50} of pyrimethamine in a concentration-dependent manner in pyrimethamine-treated-cells (from 3.10 µM without ribavirin to 1.00 µM with 4 µM of ribavirin; with CC_{50} values of 11.1 µM and 14.2 µM respectively) (Fig. 2C). Analysis of the results through the Chalice Analyzer software (Fig. S1) allowed us to calculate a synergy score of 1.49 suggesting that pyrimethamine and ribavirin interact with slight synergy (Chou, 2006).

The effect of pyrimethamine and ribavirin alone or in combination was further examined on several viral strains and species representative of the lyssavirus diversity, including RABV strains (Tha & FRA), EBLV-1 and more distantly related lyssaviruses like LBV (Delmas et al., 2008). For all viruses tested, pyrimethamine alone inhibited the viral replication with EC_{50} values < 2 µM (Fig. 3A). These EC_{50}'s further decreased (< 1 µM) when pyrimethamine is combined with 2 µM of ribavirin (Fig. 3B). For all the viruses used, EC_{50} values obtained for ribavirin alone (EC_{50} > 2 µM) are higher than that of pyrimethamine alone (Fig. 3C). The combination of ribavirin and 1 µM of pyrimethamine confirmed the synergistic effect of these drugs with an important reduction of EC_{50} (Tha: 0.36 µM; FRA: < 0.25 µM; EBLV-1: 1.9 µM & LBV: 0.5 µM) (Fig. 3D).

Thus, pyrimethamine and ribavirin alone were shown to be active at non-toxic concentrations against several species of lyssavirus, including RABV with an inhibitory potency that is amplified when used in combination.

3.3. No efficacy of pyrimethamine in vivo

The potential efficacy of pyrimethamine was then investigated in mice. By analogy with previous publications (Janse et al., 2006; Friesen et al., 2011), the pyrimethamine treatment began 3 days before inoculation by oral administration *via* the drinking water and continued for 22 days. Mice were infected with Tha RABV by i.m and were daily monitored during the experiment. Mock treated-mice were used as a control group. No significant difference in the time to onset of clinical signs or the percentage of death, was observed between the treated and mock-treated mice regardless of the viral concentration used (Fig. 4). The median lethal dose (LD_{50}) calculated was also not different between the treated-mice and the negative control (LD_{50}: 25 and 25.3 respectively) (Fig. 4). Taken together, these results showed that, in this set-up, pyrimethamine has no antiviral activity and does not provide any benefit *in vivo*. As ribavirin did not previously demonstrate any *in vivo* antiviral activities against RABV in mice (Bussereau et al., 1988) and also in humans (Warrell et al., 1989), a combination of pyrimethamine and ribavirin in mice was not attempted.

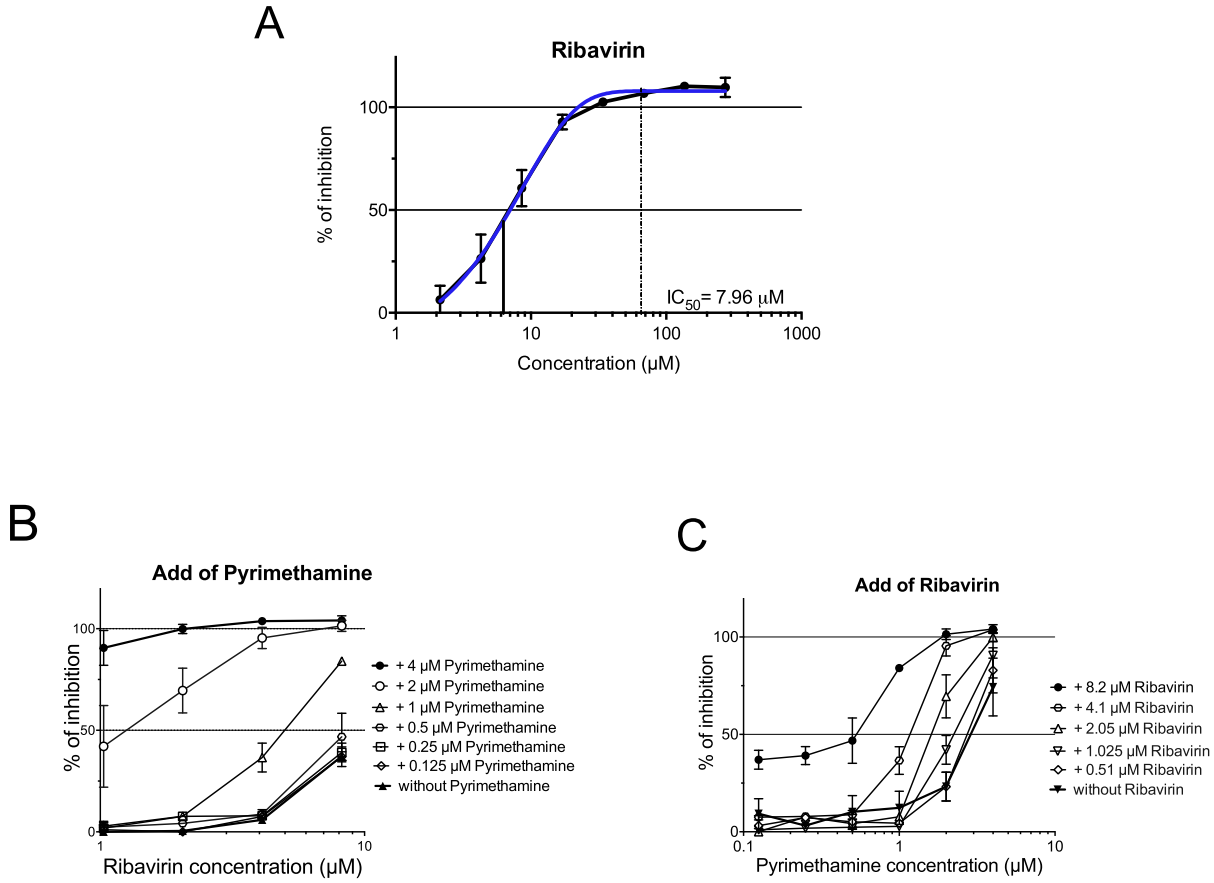

Fig. 2. Pyrimethamine and ribavirin act synergistically. **A:** BSR cells were infected with Tha isolate (200 UFF) in the presence of 0–32 µM of ribavirin. At 48 h p.i., using ELISA, percentage of inhibition of infection was calculated as the ratio of absorbance value (OD 600 nm – OD 450 nm) to the mean of absorbance values obtained from untreated-infected-cells (relative infection 100%). In addition, the toxicity of ribavirin treatment was monitored in parallel mock-infected cells using ATPlite luminescence assay system. The EC_{50} value is given and the threshold of toxicity (50% of toxicity) is indicated by the vertical dash line. **B & C:** Infected-cells (with 200 UFF of Tha) were treated with pyrimethamine (B) or Ribavirin (C) with different concentrations of ribavirin (B) or pyrimethamine (C). Dose response curves illustrate the effect of pyrimethamine on viral replication. The inhibition of rabies infection is shown as percentage of inhibition of infection relative to the DMSO treated cells. The results represent the mean and standard error of the mean (SEM) of 3 independent experiments in duplicate.

3.4. Pyrimethamine is targeting the adenosine synthesis pathway

To understand the lack of efficacy of pyrimethamine in RABV infected-mice, the action mechanisms of pyrimethamine was further investigated *in vitro*. As described before, pyrimethamine is able to cross the blood-brain barrier (McLeod et al., 1992; Weiss et al., 1988) and inhibits the *de novo* purine and thymidine synthesis pathway by blocking DHFR (Hyde, 2007). To determine whether the antiviral activities against lyssavirus observed *in vitro* is linked to the purine biosynthesis inhibition, cells transfected with Tha minigenome were treated with or without pyrimethamine in the presence of different concentrations of purine or pyrimidine nucleosides. The resulting level of minigenome replication was quantified using CAT as a reporter gene. Regardless of the condition, no significant variation of the percentage of CAT expression was noticed in DMSO-treated cells. However, addition of pyrimethamine resulted in only 30% of the CAT expression in comparison with the untreated-cells (Fig. 5). When adenosine was added, the minigenome replication was restored (72.3% of CAT expression) in pyrimethamine treated-cells, whereas guanosine, uridine and cytosine had no significant effect on CAT expression. These data indicated that antiviral activity of pyrimethamine can be partially reversed by supplementing the cell culture media with adenosine (Fig. 5).

3.5. Pyrimethamine inhibits the activation of ISRE promotor by IFNα

To better understand the biological activities of pyrimethamine and its lack of activity *in vivo*, we further investigated its capacity to inhibit the expression of IFN-inducible genes *in vitro*. To this aim, STING-37 cells stably transfected with ISRE-luciferase reporter gene (Lucas-Hourani et al., 2013) were treated with or without different concentrations of pyrimethamine. No significant difference was observed suggesting that pyrimethamine does not induce the expression of ISRE-dependent genes (Fig. 6A). Pyrimethamine did not cause toxicity in this assay except at the highest concentration (80 µM) (Fig. 6B). More interestingly, in cells treated with human recombinant IFNα, the ISRE-luciferase expression is significantly decreased in the presence of pyrimethamine compared to DMSO-treated cells (negative controls) and this inhibition was still observed with the higher dose (1000 UI/mL) of IFNα ($p < 0.01$) (Fig. 6C).

As pyrimethamine and ribavirin act synergistically on the inhibition of RABV replication and considering that ribavirin was shown to stimulate the innate immune response (Stevenson et al., 2011; Tokumoto et al., 2012), we investigated the capacity of the combined treatment to modulate the expression of ISRE-dependent genes. In the presence of exogenous IFNα, we confirmed that ribavirin alone slightly increase the ISRE-luciferase, whereas pyrimethamine alone or in combination with ribavirin significantly decreased the level of ISRE-dependent reporter gene expression at all concentrations of IFNα (range 67.5–1000 IU/mL) ($p < 0.01$) (Fig. 6D). As a control, we verified that the different treatments are non-toxic (Fig. 6E).

Altogether, this suggests that pyrimethamine alone or in combination with ribavirin inhibits the activation of the ISRE promotor by IFNα.

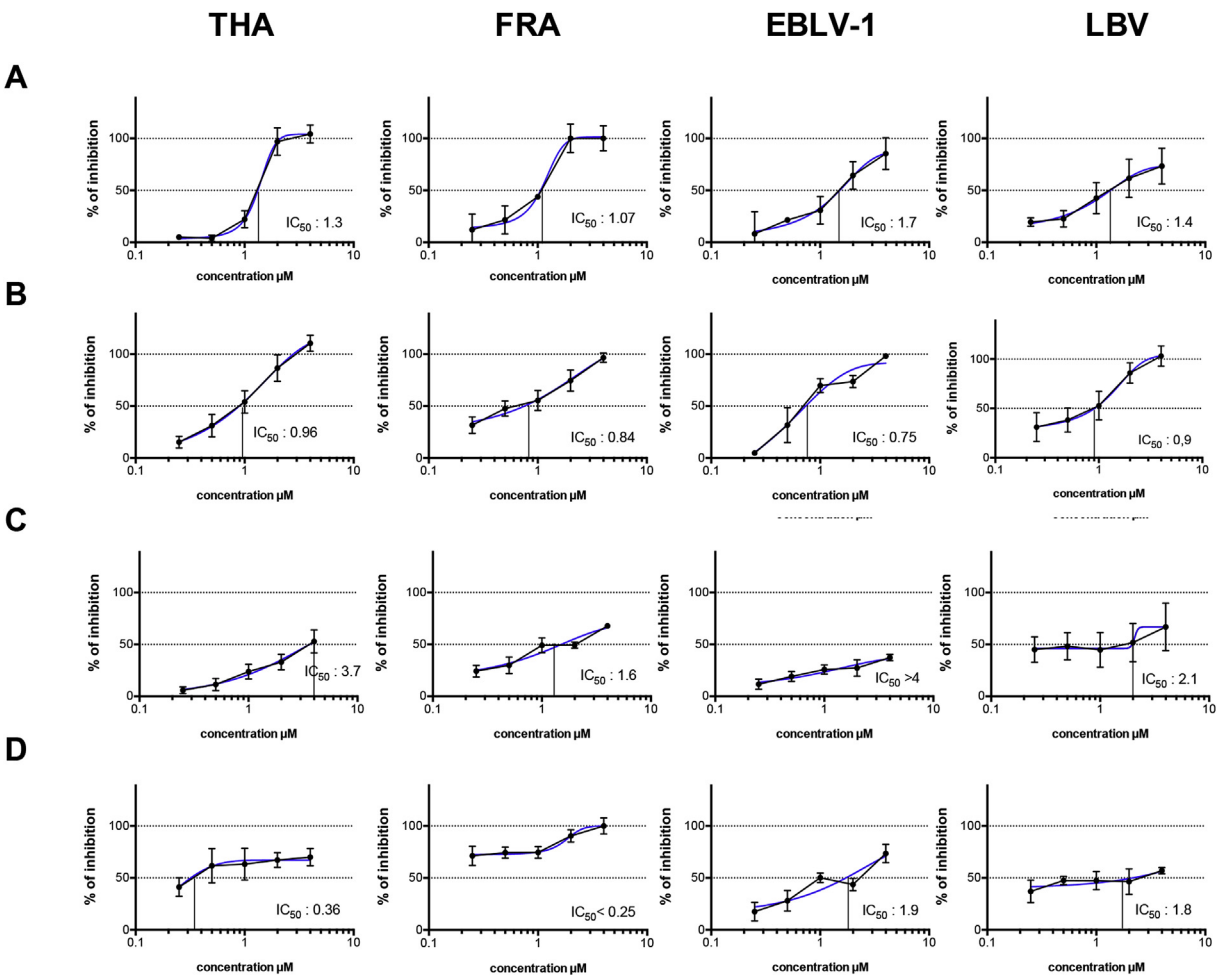

Fig. 3. Spectrum of activity of pyrimethamine and ribavirin. The spectrum of activity of pyrimethamine and ribavirin alone or in combination was tested using 4 field isolates, Tha, FRA, EBLV-1 & LVB, which belongs to 3 different species of lyssavirus. Cells were infected with 200 UFF of virus. Graphs showed the mean ± SEM of three independent experiments. **A**: Pyrimethamine at different concentrations. **B**: Pyrimethamine at different concentrations combined with 2 µM of ribavirin. C: Ribavirin at different concentrations. D: Ribavirin at different concentrations combined with 1 µM of pyrimethamine.

3.6. Pyrimethamine decreased the interferon-stimulated genes expression in infected-cells

The observations above made us believe that while pyrimethamine inhibits RABV replication, it would, *in vivo,* also inhibit the innate immune response and in that way enhance RABV replication.

To test this hypothesis, STING-37 cells were infected with an increasing MOI of Tha or CVS RABV and treated with or without pyrimethamine. As previously described, the expression of ISRE-dependent genes induced by Tha virus is very low (2.61×10^4 AU at the highest MOI), (Ben Khalifa et al., 2016). However, this activity was significantly reduced in the presence of pyrimethamine (1.9 fold with $p < 0.05$) (Fig. 7). As expected, CVS virus induced the expression of ISRE-dependent genes. The induction of ISRE-dependent gene expression reached 2.65×10^5 AU at the highest MOI but was reduced (4.7 fold) in the presence of pyrimethamine (Fig. 7). These results indicate that pyrimethamine can inhibit the activation of the ISRE promotor during RABV infection. These results were not linked to toxicity as the percentages of cell survival were similar under all conditions (data not shown).

4. Discussion

From a screening of 385 FDA-approved compounds, pyrimethamine was found to be a selective antiviral agent against RABV *in vitro*. Pyrimethamine [2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine] is currently used for the protozoal treatment such as toxoplasmic encephalitis or malaria. Pyrimethamine in association with sulfadoxine has proven to be a safe, well tolerated, efficacious and cost effective means to reduce the substantial burden of Malaria in pregnancy (Eisele et al., 2012; Fernandes et al., 2015; Kayentao et al., 2013; Ter Kuile et al., 2007; Walker et al., 2014). More recently other potential properties were reported (Corral et al., 2017). Our investigation revealed that pyrimethamine is active on different species of lyssavirus without toxicity and acts synergistically with ribavirin, an analog of guanosine which has been previously shown to have an antiviral effect against RABV *in vitro* but not *in vivo* (Appolinario and Jackson, 2015). We showed that the antiviral activity of pyrimethamine against RABV is partially linked to a low level of adenosine. We believe that a lower level of adenosine and incorporation of ribavirin instead of guanosine into the RNA causes the synergy observed in combined treatment. Our results support the parallel pathway inhibition model, which proposes that the synergy is the result of inhibition of two proteins involved in important parallel pathways for the phenotype observed (Yeh et al., 2006; Yeo et al., 2015).

Even though pyrimethamine is an effective antiviral agent against RABV *in vitro*; no benefit was observed in RABV-infected mice, confirming other recent unsuccessful attempts on experimental therapy of rabies (Dufkova et al., 2017; Marosi et al., 2018; Phoolcharoen et al., 2018; Smreczak et al., 2018). In these *in vivo* experiments,

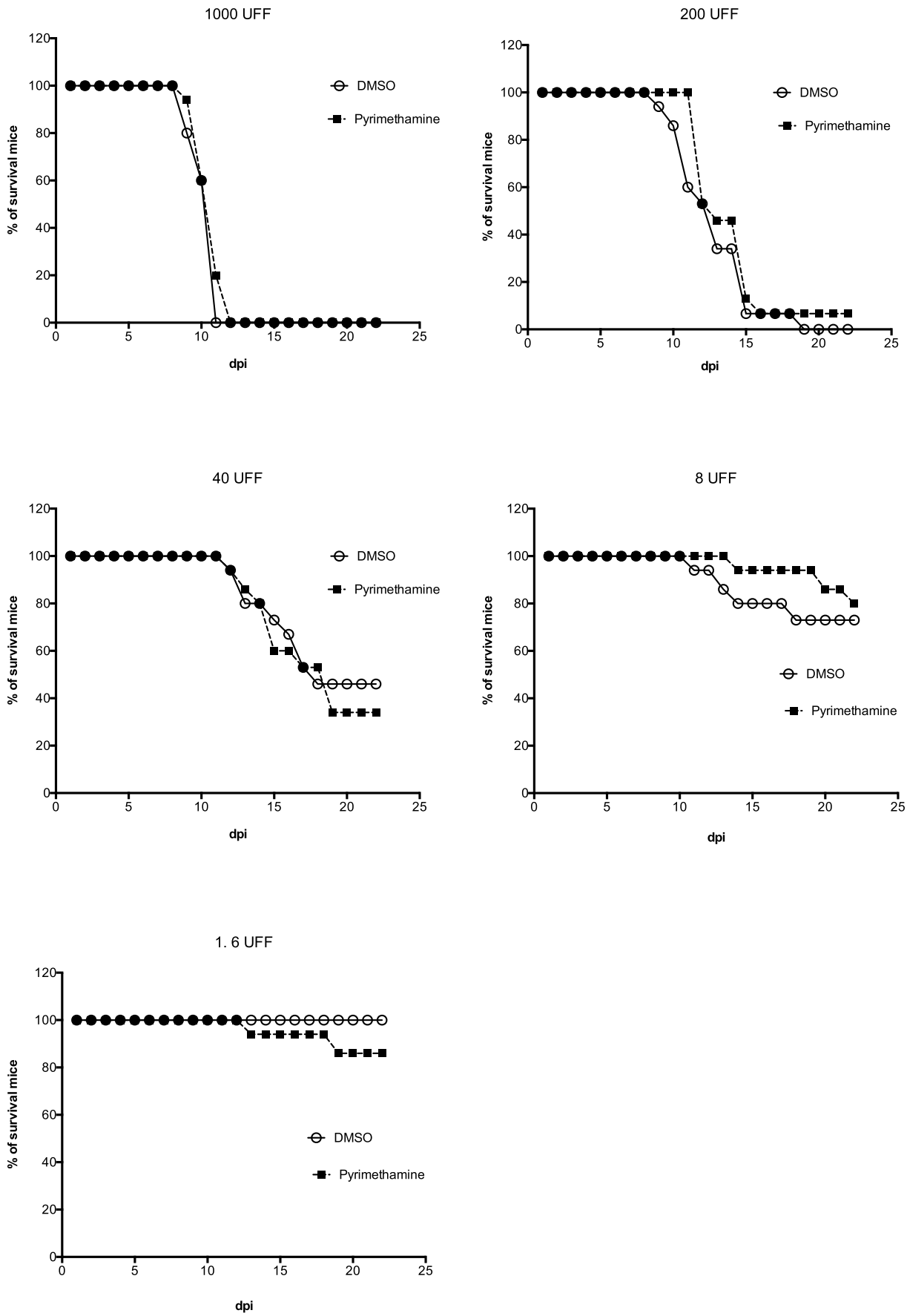

Fig. 4. Pyrimethamine has no effect in infected-mice. Groups of fifteen BALB/c mice were infected with different doses of Tha RABV by i.m. injection. Mice were treated with 70 µg/mL (281 µM) of pyrimethamine in drinking water starting 4 days before infection. Control group was treated with DMSO in the drinking water. Mice were monitored for 22 days p.i.

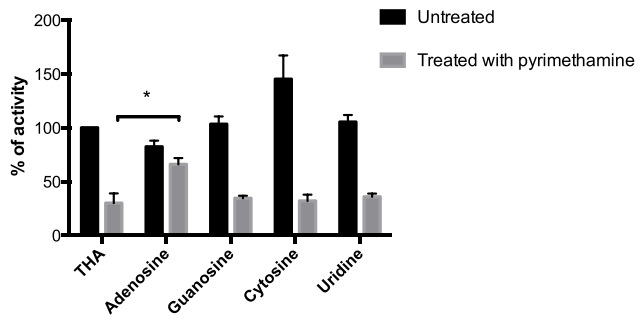

Fig. 5. Adenosine supplementation inhibits the antiviral effect of pyrimethamine. BHK-T7 cells were transfected with the minigenome based on Tha RABV. Twenty-four hours later, cells were treated with or without 100 µM of pyrimethamine and the medium was supplemented with 10 µg/mL of nucleoside. CAT expression was measured 48 h post-transfection and normalized to the control (no pyrimethamine, no added nucleoside). The results represent the mean ± SEM of three independent experiments.

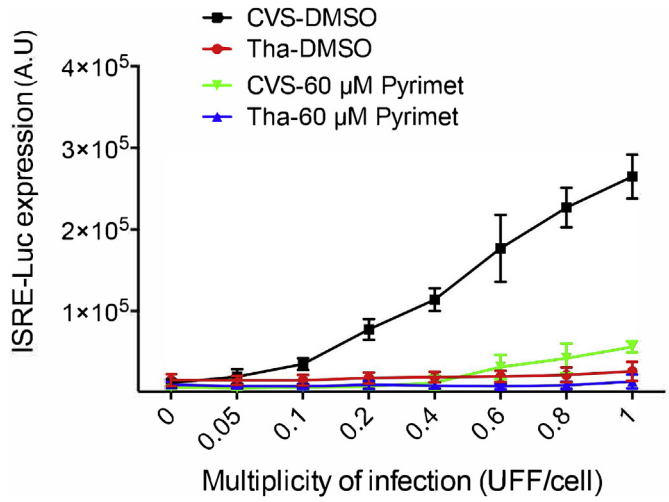

Fig. 7. Pyrimethamine reduces the induction of ISRE-gene expression. STING-37 cells were infected with increasing MOI of Tha RABV virus or with CVS virus. Twenty-four hours post-infection, cells were treated with pyrimethamine (60 µM) or DMSO and luciferase expression was measured 24 h later. The results represent the mean ± SEM of three independent experiments.

pyrimethamine was dosed and administered in the same way as in published experiments (Janse et al., 2006; Friesen et al., 2011), where it showed successful inhibition of Plasmodium replication. We are not aware of the detailed pharmacokinetics of pyrimethamine in these experiments. It is possible that the molecule accumulates to higher concentrations in cells relevant for Plasmodium replication than in cells relevant for rabies replication. In future experiments we may consider administration of pyrimethamine directly into the cerebrospinal fluid. We want to point out that pyrimethamine has been reported to cross the blood-brain barrier in humans (McLeod et al., 1992; Weiss et al., 1988).

Another explanation for the failure of the treatment in infected-mice, may be due to the concentration of adenosine in the plasma and to the purine salvage pathway. Nevertheless, different analogs of adenosine or inhibitors of purine biosynthesis are effective *in vivo* in enterovirus 71 and dengue infection (Chen et al., 2010; Deng et al., 2014). Thus, the failure of treatment by pyrimethamine *in vivo* can be only linked at

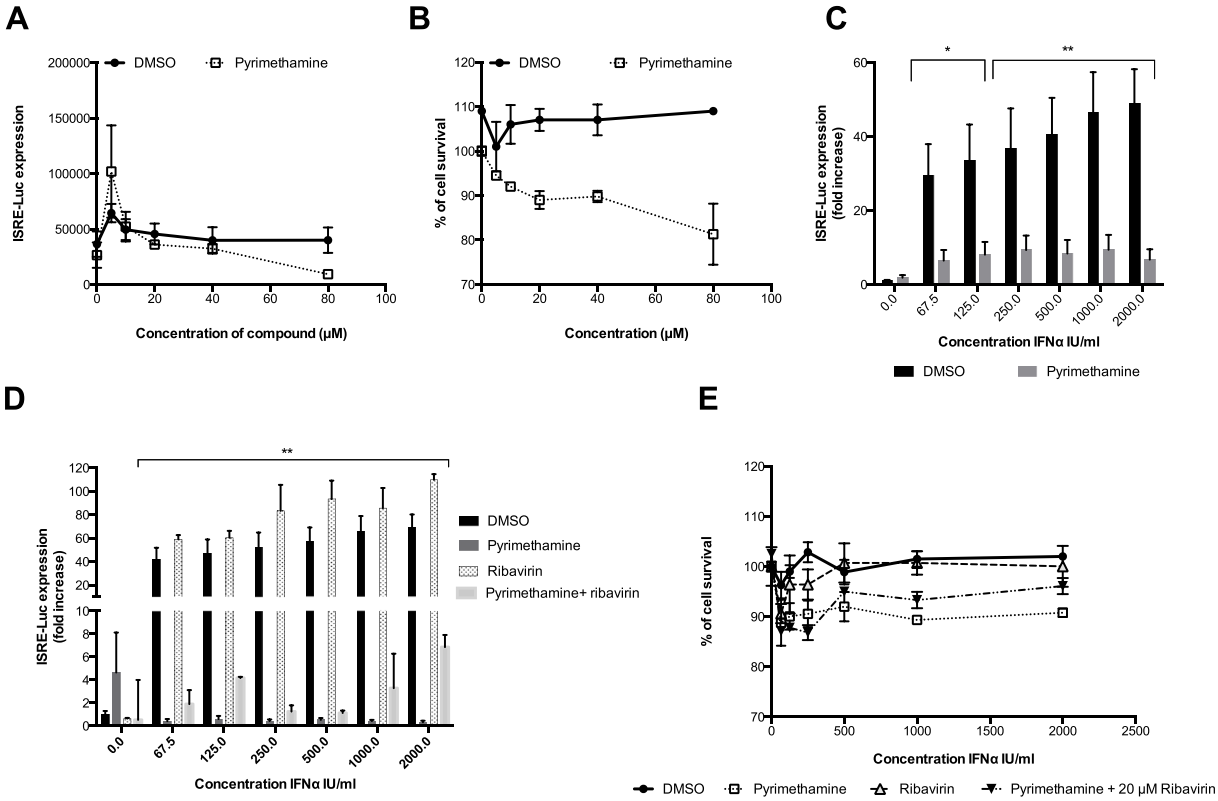

Fig. 6. Pyrimethamine reduces the induction of ISRE-gene expression. STING-37 cells, stably transfected with ISRE-luciferase reporter gene, were treated with increasing doses of pyrimethamine or DMSO. The luciferase expression was measured 24 h later (A) together with cytotoxicity (B). STING-37 cells were incubated with increasing dose of human recombinant IFNα and treated with pyrimethamine (60 µM) or DMSO (C) or with ribavirin (20 µM) with or without pyrimethamine (40 µM) (D). The fold change of ISRE-luciferase expression compared to untreated-cells (C&D) and cell viability (E) were determined 24 h later. Graphs A & D show the results of a representative experiment (n = 3). Graphs B, C & E correspond to the mean ± SEM of three independent experiments.

adenosine level.

Some antiviral mechanisms increase the expression of interferon-stimulated genes. In this regard, pyrimethamine is known to modulate the immune response (Bygbjerg, 1985, Bygbjerg et al., 1987; Takakura et al., 2011). We further analysed its role in the induction of genes under the control of ISRE promoter. We demonstrate that pyrimethamine is not stimulating ISRE gene expression and that it reduces the ISRE gene expression in non-infected-cells stimulated by IFNα, suggesting that pyrimethamine inhibits the effect of IFN on ISRE-gene expression. Thus, reduction of ISRE-gene expression could be linked to the inhibition of STAT pathways by pyrimethamine. Indeed, it is known that pyrimethamine can modulate STAT signaling pathway by reducing phosphorylation of STAT3 (Takakura et al., 2011). The replication and spread of RABV in the CNS are controlled by immune mechanisms, and early expression of type I IFN seems to be important for survival or the delay of mortality (Barkhouse et al., 2015; Chopy et al., 2011; Wang et al., 2005). However, rabies virus blocked type I IFN signaling (Brzózka et al., 2006; Chelbi-Alix et al., 2006; Wiltzer et al., 2012) and our work shows that the propagation of the virus could be facilitated by pyrimethamine. This may allow rabies to escape the innate immune response in our *in vivo* experiments.

In conclusion, our work reveals that use of pyrimethamine, an inhibitor of purine synthesis, does not allow survival of rabies-infected mice. This lack of *in vivo* efficacy could be linked to the salvage of purine pathway and to the inhibitory effect of pyrimethamine on the innate immune response.

Fundings

This project was supported by the European Union's Seventh Framework Program under grant agreement no. 260644-SILVER.

Conflicts of interest

None declared.

Acknowledgement

We are grateful to Pr. Karl Klaus Conzelmann for providing us plasmids used to develop minigenome system.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.10.016.

References

Appolinario, C.M., Jackson, A.C., 2015. Antiviral therapy for human rabies. Antivir. Ther. 20, 1–10. https://doi.org/10.3851/IMP2851.

Assenberg, R., Delmas, O., Morin, B., Graham, S.C., De Lamballerie, X., Laubert, C., Coutard, B., Grimes, J.M., Neyts, J., Owens, R.J., Brandt, B.W., Gorbalenya, A., Tucker, P., Stuart, D.I., Canard, B., Bourhy, H., 2010. Genomics and structure/function studies of Rhabdoviridae proteins involved in replication and transcription. Antivir. Res. 87, 149–161. https://doi.org/10.1016/j.antiviral.2010.02.322.

Barkhouse, D.A., Garcia, S.A., Bongiorno, E.K., Lebrun, A., Faber, M., Hooper, D.C., 2015. Expression of interferon gamma by a recombinant rabies virus strongly attenuates the pathogenicity of the virus via induction of type I interferon. J. Virol. 89, 312–322. https://doi.org/10.1128/JVI.01572-14.

Ben Khalifa, Y., Luco, S., Besson, B., Sonthonnax, F., Archambaud, M., Grimes, J.M., Larrous, F., Bourhy, H., 2016. The matrix protein of rabies virus binds to RelAp43 to modulate NF-κB-dependent gene expression related to innate immunity. Sci. Rep. 21 (6), 39420. https://doi.org/10.1038/srep39420.

Bourhy, H., Goudal, M., Mailles, A., Sadkowska-Todys, M., Dacheux, L., Zeller, H., 2009. Is there a need for anti-rabies vaccine and immunoglobulins rationing in Europe? Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 14.

Bourhy, H., Dautry-Varsat, A., Hotez, P.J., Salomon, J., 2010. Rabies, still neglected after 125 years of vaccination. PLoS Neglected Trop. Dis. 4, e839. https://doi.org/10.1371/journal.pntd.0000839.

Brzózka, K., Finke, S., Conzelmann, K.-K., 2006. Inhibition of interferon signaling by rabies virus phosphoprotein P: activation-dependent binding of STAT1 and STAT2. J. Virol. 80, 2675–2683. https://doi.org/10.1128/JVI.80.6.2675-2683.2006.

Buchholz, U.J., Finke, S., Conzelmann, K.K., 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. 73, 251–259.

Bussereau, F., Picard, M., Blancou, J., Sureau, P., 1988. Treatment of rabies in mice and foxes with antiviral compounds. Acta Virol. 32, 33–49.

Bygbjerg, I.C., 1985. Pyrimethamine-induced alterations in human lymphocytes *in vitro*. Mechanisms and reversal of the effect. Acta Pathol. Microbiol. Immunol. Scand. 93, 183–188.

Bygbjerg, I.C., Svenson, M., Theander, T.G., Bendtzen, K., 1987. Effect of antimalarial drugs on stimulation and interleukin 2 production of human lymphocytes. Int. J. Immunopharm. 9, 513–519.

Chelbi-Alix, M.K., Vidy, A., El Bougrini, J., Blondel, D., 2006. Rabies viral mechanisms to escape the IFN system: the viral protein P interferes with IRF-3, Stat1, and PML nuclear bodies. J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res. 26, 271–280. https://doi.org/10.1089/jir.2006.26.271.

Chen, Y.-L., Yin, Z., Lakshminarayana, S.B., Qing, M., Schul, W., Duraiswamy, J., Kondreddi, R.R., Goh, A., Xu, H.Y., Yip, A., Liu, B., Weaver, M., Dartois, V., Keller, T.H., Shi, P.-Y., 2010. Inhibition of dengue virus by an ester prodrug of an adenosine analog. Antimicrob. Agents Chemother. 54, 3255–3261. https://doi.org/10.1128/AAC.00397-10.

Chopy, D., Detje, C.N., Lafage, M., Kalinke, U., Lafon, M., 2011. The type I interferon response bridles rabies virus infection and reduces pathogenicity. J. Neurovirol. 17, 353–367. https://doi.org/10.1007/s13365-011-0041-6.

Chou, T.-C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621–681. https://doi.org/10.1124/pr.58.3.10.

Corral, M.G., Leroux, J., Stubbs, K.A., Mylne, J.S., 2017. Herbicidal properties of antimalarial drugs. Sci. Rep. 7, 45871. https://doi.org/10.1038/srep45871.

Coutard, B., Gorbalenya, A.E., Snijder, E.J., Leontovich, A.M., Poupon, A., De Lamballerie, X., Charrel, R., Gould, E.A., Gunther, S., Norder, H., Klempa, B., Bourhy, H., Rohayem, J., L'hermite, E., Nordlund, P., Stuart, D.I., Owens, R.J., Grimes, J.M., Tucker, P.A., Bolognesi, M., Mattevi, A., Coll, M., Jones, T.A., Aqvist, J., Unge, T., Hilgenfeld, R., Bricogne, G., Neyts, J., La Colla, P., Puerstinger, G., Gonzalez, J.P., Leroy, E., Cambillau, C., Romette, J.L., Canard, B., 2008. The VIZIER project: preparedness against pathogenic RNA viruses. Antivir. Res. 78, 37–46. https://doi.org/10.1016/j.antiviral.2007.10.013.

Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., Cameron, C.E., 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat. Med. 6, 1375–1379. https://doi.org/10.1038/82191.

Dacheux, L., Wacharapluesadee, S., Hemachudha, T., Meslin, F.-X., Buchy, P., Reynes, J.-M., Bourhy, H., 2010. More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. PLoS Neglected Trop. Dis. 4, e765. https://doi.org/10.1371/journal.pntd.0000765.

De Benedictis, P., Minola, A., Rota Nodari, E., Aiello, R., Zecchin, B., Salomoni, A., et al., 2016. Development of broad-spectrum human monoclonal antibodies for rabies post-exposure prophylaxis. EMBO Mol. Med. 8 (4), 407.

Delmas, O., Holmes, E.C., Talbi, C., Larrous, F., Dacheux, L., Bouchier, C., Bourhy, H., 2008. Genomic diversity and evolution of the lyssaviruses. PloS One 3. https://doi.org/10.1371/journal.pone.0002057.

Deng, C.-L., Yeo, H., Ye, H.-Q., Liu, S.-Q., Shang, B.-D., Gong, P., Alonso, S., Shi, P.-Y., Zhang, B., 2014. Inhibition of enterovirus 71 by adenosine analog NITD008. J. Virol. 88, 11915–11923. https://doi.org/10.1128/JVI.01207-14.

Dodet, B., Africa Rabies Bureau (AfroREB), 2009. The fight against rabies in Africa: from recognition to action. Vaccine 27, 5027–5032. https://doi.org/10.1016/j.vaccine.2009.06.030.

Dufkova, L., Sirmarova, J., Salat, J., Honig, V., Palus, M., Ruzek, D., Fooks, A.R., Mansfield, K.L., Tordo, N., Jochmans, D., Neyts, J., Martina, B., Koraka, P., Osterhaus, A.D.M.E., ASKLEPIOS consortium, 2017. Mannitol treatment is not effective in therapy of rabies virus infection in mice. Vaccine. https://doi.org/10.1016/j.vaccine.2017.12.028.

Eisele, T.P., Larsen, D.A., Anglewicz, P.A., Keating, J., Yukich, J., Bennett, A., Hutchinson, P., Steketee, R.W., 2012. Malaria prevention in pregnancy, birthweight, and neonatal mortality: a meta-analysis of 32 national cross-sectional datasets in Africa. Lancet Infect. Dis. 12, 942–949. https://doi.org/10.1016/S1473-3099(12)70222-0.

Fernandes, S., Sicuri, E., Kayentao, K., van Eijk, A.M., Hill, J., Webster, J., Were, V., Akazili, J., Madanitsa, M., ter Kuile, F.O., Hanson, K., 2015. Cost-effectiveness of two versus three or more doses of intermittent preventive treatment for malaria during pregnancy in sub-Saharan Africa: a modelling study of meta-analysis and cost data. Lancet Glob. Health 3, e143–153. https://doi.org/10.1016/S2214-109X(14)70385-7.

Fooks, A.R., Cliquet, F., Finke, S., Freuling, C., Hemachudha, T., Mani, R.S., Müller, T., Nadin-Davis, S., Picard-Meyer, E., Wilde, H., Banyard, A.C., 2017. Rabies. Nat. Rev. Dis. Primers 3, 17091. https://doi.org/10.1038/nrdp.2017.91. 2017 Nov 30.

Friesen, J., Borrmann, S., Matuschewski, K., 2011. Induction of antimalaria immunity by pyrimethamine prophylaxis during exposure to sporozoites is curtailed by parasite resistance. Antimicrob. Agents Chemother. 55, 2760–2767. https://doi.org/10.1128/AAC.01717-10.

Ghanem, A., Kern, A., Conzelmann, K.-K., 2012. Significantly improved rescue of rabies virus from cDNA plasmids. Eur. J. Cell Biol. 91, 10–16. https://doi.org/10.1016/j.ejcb.2011.01.008.

Hampson, K., Coudeville, L., Lembo, T., Sambo, M., Kieffer, A., Attlan, M., Barrat, J., Blanton, J.D., Briggs, D.J., Cleaveland, S., Costa, P., Freuling, C.M., Hiby, E., Knopf, L., Leanes, F., Meslin, F.-X., Metlin, A., Miranda, M.E., Müller, T., Nel, L.H., Recuenco, S., Rupprecht, C.E., Schumacher, C., Taylor, L., Vigilato, M.A.N., Zinsstag, J., Dushoff, J., Global Alliance for Rabies Control Partners for Rabies Prevention, 2015.

Estimating the global burden of endemic canine rabies. PLoS Negl. Trop. Dis. 9, e0003709. https://doi.org/10.1371/journal.pntd.0003709.
Hemachudha, T., Sunsaneewitayakul, B., Desudchit, T., Suankratay, C., Sittipunt, C., Wacharapluesadee, S., Khawplod, P., Wilde, H., Jackson, A.C., 2006. Failure of therapeutic coma and ketamine for therapy of human rabies. J. Neurovirol. 12, 407–409. https://doi.org/10.1080/13550280600902295.
Hyde, J.E., 2007. Targeting purine and pyrimidine metabolism in human apicomplexan parasites. Curr. Drug Targets 8, 31–47.
Jackson, A.C., 2013. Current and future approaches to the therapy of human rabies. Antivir. Res. 99, 61–67. https://doi.org/10.1016/j.antiviral.2013.01.003.
Jackson, A.C., 2012. Is minocycline useful for therapy of acute viral encephalitis? Antivir. Res. 95, 242–244. https://doi.org/10.1016/j.antiviral.2012.06.005.
Janse, C.J., Ramesar, J., Waters, A.P., 2006. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat. Protoc. 1, 346–356. https://doi.org/10.1038/nprot.2006.53.
Kayentao, K., Garner, P., van Eijk, A.M., Naidoo, I., Roper, C., Mulokozi, A., MacArthur, J.R., Luntamo, M., Ashorn, P., Doumbo, O.K., ter Kuile, F.O., 2013. Intermittent preventive therapy for malaria during pregnancy using 2 vs 3 or more doses of sulfadoxine-pyrimethamine and risk of low birth weight in Africa: systematic review and meta-analysis. JAMA 309, 594–604. https://doi.org/10.1001/jama.2012.216231.
Lucas-Hourani, M., Dauzonne, D., Jorda, P., Cousin, G., Lupan, A., Helynck, O., Caignard, G., Janvier, G., André-Leroux, G., Khiar, S., Escriou, N., Desprès, P., Jacob, Y., Munier-Lehmann, H., Tangy, F., Vidalain, P.-O., 2013. Inhibition of pyrimidine biosynthesis pathway suppresses viral growth through innate immunity. PLoS Pathog. 9, e1003678. https://doi.org/10.1371/journal.ppat.1003678.
Marosi, A., Dufkova, L., Forró, B., Felde, O., Erdélyi, K., Širmarová, J., Palus, M., Hönig, V., Salát, J., Tikos, R., Gyuranecz, M., Růžek, D., Martina, B., Koraka, P., Osterhaus, A.D.M.E., Bakonyi, T., 2018. Combination therapy of rabies-infected mice with inhibitors of pro-inflammatory host response, antiviral compounds and human rabies immunoglobulin. Vaccine. https://doi.org/10.1016/j.vaccine.2018.05.066.
McLeod, R., Mack, D., Foss, R., Boyer, K., Withers, S., Levin, S., Hubbell, J., 1992. Levels of pyrimethamine in sera and cerebrospinal and ventricular fluids from infants treated for congenital toxoplasmosis. toxoplasmosis study group. Antimicrob. Agents Chemother. 36, 1040–1048.
Phoolcharoen, W., Banyard, A.C., Prehaud, C., Selden, D., Wu, G., Birch, C.P.D., Szeto, T.H., Lafon, M., Fooks, A.R., Ma, J.K.-C., 2018. In vitro and in vivo evaluation of a single chain antibody fragment generated in planta with potent rabies neutralisation activity. Vaccine. https://doi.org/10.1016/j.vaccine.2018.02.057.
Ribadeau-Dumas, F., Cliquet, F., Gautret, P., Robardet, E., Le Pen, C., Bourhy, H., 2016. Travel-associated rabies in pets and residual rabies risk, Western Europe. Emerg. Infect. Dis. 22, 1268–1271. https://doi.org/10.3201/eid2207.151733.
Santos, A., Cale, E., Dacheux, L., Bourhy, H., Gouveia, J., Vasconcelos, P., 2012. Fatal case of imported human rabies in Amadora, Portugal, August 2011. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 17.
Shantavasinkul, P., Wilde, H., 2011. Postexposure prophylaxis for rabies in resource-limited/poor countries. Adv. Virus Res. 79, 291–307. https://doi.org/10.1016/B978-0-12-387040-7.00013-5.
Smreczak, M., Orłowska, A., Marzec, A., Trębas, P., Kycko, A., Reichert, M., Koraka, P., Osterhaus, A.D.M.E., Żmudziński, J.F., 2018. The effect of combined drugs therapy on the course of clinical rabies infection in a murine model. Vaccine. https://doi.org/10.1016/j.vaccine.2018.04.003.
Stevenson, N.J., Murphy, A.G., Bourke, N.M., Keogh, C.A., Hegarty, J.E., O'Farrelly, C., 2011. Ribavirin enhances IFN-α signalling and MxA expression: a novel immune modulation mechanism during treatment of HCV. PloS One 6, e27866. https://doi.org/10.1371/journal.pone.0027866.
Superti, F., Seganti, L., Pana, A., Orsi, N., 1985. Effect of amantadine on rhabdovirus infection. Drugs Exp. Clin. Res. 11 (1), 69–74 Epub 1985/01/01. PubMed PMID: 3013559.
Takakura, A., Nelson, E.A., Haque, N., Humphreys, B.D., Zandi-Nejad, K., Frank, D.A., Zhou, J., 2011. Pyrimethamine inhibits adult polycystic kidney disease by modulating STAT signaling pathways. Hum. Mol. Genet. 20, 4143–4154. https://doi.org/10.1093/hmg/ddr338.
Tam, R.C., Lau, J.Y.N., Hong, Z., 2001. Mechanisms of action of ribavirin in antiviral therapies. Antivir. Chem. Chemother. 12, 261–272.
Ter Kuile, F.O., van Eijk, A.M., Filler, S.J., 2007. Effect of sulfadoxine-pyrimethamine resistance on the efficacy of intermittent preventive therapy for malaria control during pregnancy: a systematic review. JAMA 297, 2603–2616. https://doi.org/10.1001/jama.297.23.2603.
Thongcharoen, P., Sureau, P., Wasi, C., Bourhy, H., Chaiprasithikul, P., Puthavathana, P., 1990. Monoclonal antibody studies of rabies viruses isolated from Thailand. Southeast Asian J. Trop. Med. Public Health 21, 129–133.
Tokumoto, Y., Hiasa, Y., Uesugi, K., Watanabe, T., Mashiba, T., Abe, M., Kumagi, T., Ikeda, Y., Matsuura, B., Onji, M., 2012. Ribavirin regulates hepatitis C virus replication through enhancing interferon-stimulated genes and interleukin 8. J. Infect. Dis. 205, 1121–1130. https://doi.org/10.1093/infdis/jis025.
Walker, P.G.T., ter Kuile, F.O., Garske, T., Menendez, C., Ghani, A.C., 2014. Estimated risk of placental infection and low birthweight attributable to Plasmodium falciparum malaria in Africa in 2010: a modelling study. Lancet Glob. Health 2, e460–467. https://doi.org/10.1016/S2214-109X(14)70256-6.
Wang, Z.W., Sarmento, L., Wang, Y., Li, X., Dhingra, V., Tseggai, T., Jiang, B., Fu, Z.F., 2005. Attenuated rabies virus activates, while pathogenic rabies virus evades, the host innate immune responses in the central nervous system. J. Virol. 79, 12554–12565. https://doi.org/10.1128/JVI.79.19.12554-12565.2005.
Warrell, M.J., White, N.J., Looareesuwan, S., Phillips, R.E., Suntharasamai, P., Chanthavanich, P., Riganti, M., Fisher-Hoch, S.P., Nicholson, K.G., Manatsathit, S., 1989. Failure of interferon alfa and tribavirin in rabies encephalitis. BMJ 299, 830–833.
Weiss, L.M., Harris, C., Berger, M., Tanowitz, H.B., Wittner, M., 1988. Pyrimethamine concentrations in serum and cerebrospinal fluid during treatment of acute Toxoplasma encephalitis in patients with AIDS. J. Infect. Dis. 157, 580–583.
WHO, 2017. Human rabies: 2016 updates and call for data. WHO [WWW Document]. http://www.who.int/rabies/resources/who_wer9207/en/, Accessed date: 19 April 2017.
WHO Rabies vaccines: 2018 WHO position paper – April 2018 WHO URl http://origin.who.int/rabies/resources/who_wer9316/en/(accessed 4.20.2018).
Wilde, H., Lumlertdacha, B., 2011. Rabies research in resource-poor countries. Adv. Virus Res. 79, 449–455. https://doi.org/10.1016/B978-0-12-387040-7.00021-4.
Willoughby Jr., R.E., Hammarin, A.L., 2005. Prophylaxis against rabies in children exposed to bats. Pediatr. Infect. Dis. J. 24 (12), 1109–1110 Epub 2005/12/24. PubMed PMID: 16371876.
Wiltzer, L., Larrous, F., Oksayan, S., Ito, N., Marsh, G.A., Wang, L.F., Blondel, D., Bourhy, H., Jans, D.A., Moseley, G.W., 2012. Conservation of a unique mechanism of immune evasion across the Lyssavirus genus. J. Virol. 86, 10194–10199. https://doi.org/10.1128/JVI.01249-12.
Yamada, K., Noguchi, K., Komeno, T., Furuta, Y., Nishizono, A., 2016. Efficacy of Favipiravir (T-705) in rabies postexposure prophylaxis. J. Infect. Dis. 213, 1253–1261. https://doi.org/10.1093/infdis/jiv586.
Yeh, L.-C.C., Zavala, M.C., Lee, J.C., 2006. C-type natriuretic peptide enhances osteogenic protein-1-induced osteoblastic cell differentiation via Smad5 phosphorylation. J. Cell. Biochem. 97, 494–500. https://doi.org/10.1002/jcb.20657.
Yeo, K.L., Chen, Y.-L., Xu, H.Y., Dong, H., Wang, Q.-Y., Yokokawa, F., Shi, P.-Y., 2015. Synergistic suppression of dengue virus replication using a combination of nucleoside analogs and nucleoside synthesis inhibitors. Antimicrob. Agents Chemother. 59, 2086–2093. https://doi.org/10.1128/AAC.04779-14.