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Pyrimethamine inhibits rabies virus replication in vitro

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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 e
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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.

Rabies virus replication can be inhibited by pyrimethamine. Antiviral susceptibility is lost due to the development of drug resistant mutants. This suggests that pyrimethamine needs to be used in combination with other antivirals. In human rabies, 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 (Dodeet 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 Lumleartdachy, 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
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 (Busseareau 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 unpromising, disappointing results.

Recently Favipiravir, a broad spectrum RNA-virus inhibitor, was found to have moderate activity on RABV in vitro (EC₅₀ ~ 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-Hounari et al., 2013). These are HEK293 cells stably transfected by a reporter plasmid carrying the luciferase gene under the control of interferon-stimulated response elements (ISRE). STING-37 cells, grown in Glasgow medium supplemented with 10% calf serum, tryptose phosphate, non-essential amino acids and antibiotics. STING-37 cells were transfected with pCMV-RL (Promega) according to manufacturer's instruction on undiluted BSR cell lysate. Non treated-cells infected by Tha virus were used as a positive control, one of the pTIT vectors was omitted. Forty-eight hours post transfection, cells were harvested in passive lysis buffer (Promega) according to manufacturer's instruction (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 protocol. The antiviral 50% effective concentration (EC₅₀) and the 50% cytotoxic concentration (CC₅₀) were calculated by nonlinear regression analysis. Each experiment was 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 $1 \times 10^6$ 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 (Franc, 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 (14401 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 diaminopyrimidine derivative that inhibits the dihydrofolate reductase (DHFR) and blocks de novo purine synthesis.

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 efficacity (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. 51) 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.
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. 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 EC50 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.
3.6. 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 × 10⁴ 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 × 10⁵ 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 (Duřková 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.
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
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; Choppy 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.

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Conflicts of interest

None declared.

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Appendix A. Supplementary data

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

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