Synergistic antiviral activity of ribavirin and interferon-α against parrot bornaviruses in avian cells

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Avian bornaviruses are the causative agents of proventricular dilatation disease (PDD), a widely distributed and often fatal disease in captive psittacines. Because neither specific prevention measures nor therapies against PDD and bornavirus infections are currently available, new antiviral strategies are required to improve animal health. We show here that the nucleoside analogue ribavirin inhibited bornavirus activity in a polymerase reconstitution assay and reduced viral load in avian cell lines infected with two different parrot bornaviruses. Furthermore, we observed that ribavirin enhanced type I IFN signalling in avian cells. Combined treatment of avian bornavirus-infected cells with ribavirin and recombinant IFN-α strongly enhanced the antiviral efficiency compared to either drug alone. The combined use of ribavirin and type I IFN might represent a promising new strategy for therapeutic treatment of captive parrots persistently infected with avian bornaviruses.
Activity of ribavirin against avian bornaviruses

against avian bornaviruses is an important step towards improving animal health.

In a previous study, we showed that recombinant chicken IFN-α (chIFN-α) efficiently inhibited PaBV-2 and PaBV-4 in infected quail cells (Reuter et al., 2010). However, due to the lack of homologous IFN preparations, no in vivo data on the efficacy of IFN in infected psittacines are available. The nucleoside analogue ribavirin is active against a broad range of RNA viruses (Day et al., 2005; Graci et al., 2007; Gu et al., 2006; Leyssen et al., 2005; Moreno et al., 2011; Pauly & Lauring, 2015; Severson et al., 2003), including the mammalian Borna disease virus 1 (BoDV-1), which is a close relative of the avian bornaviruses (Kuhn et al., 2015). Several studies demonstrated that ribavirin can inhibit BoDV-1 in mammalian cell lines (Jordon et al., 1999; Mizutani et al., 1998, 1999) as well as in experimentally infected gerbils (Lee et al., 2008). More recently, a similar antiviral effect was described also for duck embryo fibroblast (DEF) cultures freshly infected with PaBV-4 (Musser et al., 2015).

To confirm and extend these observations, we used QM7 quail cell cultures persistently infected with either isolate PaBV-2 #6609 or PaBV-4 #6758 (Rinder et al., 2009) which were grown in medium containing 1 or 5 µg ml⁻¹ of ribavirin (Rebetol; MSD Sharp & Dohme) and passaged twice weekly. The proportion of infected cells was assessed by immunofluorescence using a polyclonal rabbit serum detecting the viral N protein (Reuter et al., 2010; Zimmermann et al., 2014). After 5 days of treatment with either dose of ribavirin, the number of infected cells had already decreased substantially (Fig. 1a). At day 15, only a few cells still expressed the viral N protein at detectable levels, and the virus was seemingly eliminated after 4 weeks of treatment (Fig. 1b). However, when the ribavirin treatment was stopped after 29 days, the number of virus-positive cells steadily increased to almost 100 % within 2–3 weeks (Fig. 1b). Similar antiviral effects of ribavirin were observed when acute infections were mimicked by mixing a low number of infected cells with an excess of uninfected cells (Fig. 1c). While the proportion of PaBV-infected cells steadily increased in untreated cultures, PaBV N-positive cells remained barely detectable in ribavirin-treated cultures. When the treatment was stopped on day 18, PaBV-2 #6609 quickly emerged and infected virtually all cells of the culture within 10 days. PaBV-4 #6758 replicated far less efficiently under these conditions and, as a consequence, the antiviral effect of ribavirin was stronger, but an elimination of the virus was not achieved (Fig. 1c).

Ribavirin may inhibit virus replication by reducing the cellular GTP pool through inhibition of the inosine monophosphate dehydrogenase (Leyssen et al., 2005; Mori et al., 2011). In cell culture, this effect can be counteracted by adding exogenous guanosine, which is converted into GTP after cellular uptake (Jordan et al., 1999; Olschlager et al., 2011). Therefore, persistently infected QM7 cells were treated with 1 µg ml⁻¹ of ribavirin and, additionally, with an excess of guanosine (Sigma-Aldrich). To control for the possibility that guanosine simply interfered with ribavirin uptake, some cultures were also treated with adenosine (Sigma-Aldrich), which is expected to compete with ribavirin for uptake, but is unable to replenish the GTP pool (Fukuchi et al., 2010). We found that addition of either guanosine or adenosine reduced the antiviral activity of ribavirin in PaBV-4-infected QM7 cells to similar extents (Fig. 2a), suggesting that this is due to competitive inhibition of cellular ribavirin uptake by both compounds. This result does not agree with a previous study which found that the ribavirin-induced antiviral activity was inhibited by guanosine but not adenosine supplementation in BoDV-1-infected human oligodendroglia (OL) cells (Jordan et al., 1999). The discrepancy between our results and those of Jordan et al. (1999) may be due to different mechanisms involved in the inhibition of BoDV-1 and PaBV-4 or due to variations in the extent of competitive inhibition of ribavirin uptake into human OL and quail QM7 cells. In another study, Musser et al. (2015) showed that supplementation of growth media with guanosine can partly prevent the antiviral effect of ribavirin in PaBV-4-infected DEF cultures, but competition for cellular uptake by adenosine was not tested. Neither our results nor the results of Musser et al. (2015) allow conclusions on the inhibition of avian bornaviruses by ribavirin-induced reduction of intracellular GTP pools. In both studies, the antagonism of ribavirin by guanosine supplementation may have been the result of replenished GTP pools as well as of competitive reduction of intracellular ribavirin levels.

A second mechanism of ribavirin action is that the drug is incorporated into viral RNA, thereby introducing mutations (Day et al., 2005; Gu et al., 2006; Moreno et al., 2011; Pauly & Lauring, 2015; Severson et al., 2003). To test this possibility, QM7 cells persistently infected with PaBV-4 #6758 were treated with 1 or 5 µg ml⁻¹ of ribavirin during passaging for 3 weeks. Detection of bornavirus N protein-positive cells and quantification of PaBV-4 phosphoprotein (P) gene RNA copies using a specific quantitative PCR (Honkavuori et al., 2008; Rubbenstroth et al., 2014) confirmed the efficiency of the treatment (Fig. 2b, c). After 5, 11 and 22 days of treatment, cDNA was generated by Transcriptor High Fidelity cDNA Synthesis kit (Roche Life Sciences). Three PCR fragments of 981–1304 bp length covering the N, accessory protein X and P genes and parts of the RNA-dependent RNA polymerase (L) gene were amplified using iProof High-Fidelity DNA Polymerase (Bio-Rad). Primer pairs were designed to target cDNA derived from genomic PaBV-4 RNA but not from mRNA transcripts (Fig. S1, available in the online Supplementary Material). The amplicons were pooled in equimolar amounts and Illumina compatible libraries were prepared as described previously (Juozapaitis et al., 2014). The resultant libraries were sequenced on an Illumina MiSeq instrument in 2×300 bp PE mode using a MiSeq Reagent kit v3 600 cycle (Illumina). Mean sequence variation frequencies per nucleotide position from the PaBV-4 #6758 reference sequence (JX065209) were calculated. Variations detected in
all ribavirin-treated and non-treated samples were considered to originate from viral subpopulations present in the persistently infected QM7 culture prior to the beginning of the experiment. Thus, these shared variations were excluded from the analysis. Mean variation frequencies were approximately 0.1% per nucleotide position in all samples, irrespective of treatment (Fig. 2d). It is possible that these variations are technical artefacts resulting from errors during reverse transcription or PCR amplification rather than from mutations generated during viral replication. Indeed, the low frequency of variation and the lack of a marked increase under ribavirin treatment (Fig. 2d) strongly suggest that hypermutation of the viral genome is probably not the major mode of ribavirin action against avian bornaviruses.

Ribavirin can directly inhibit the activities of viral polymerases (Arias et al., 2008; Hofmann et al., 2008; Parker, 2005; Pfeiffer & Kirkegaard, 2005; Te et al., 2007; Young et al., 2003). To measure such effects, we established a PaBV-4 polymerase reconstitution assay in chicken fibroblast DF-1 cells as described previously for BoDV-1 (Kojima et al., 2014; Yanai et al., 2006). Briefly, the L polymerase, N and P genes of PaBV-4 #6758 (GenBank accession number JX065209) and a bornavirus minigenome were co-expressed from separate plasmids under the control

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Fig. 1. Ribavirin inhibits PaBV infection in cell culture but does not eliminate the virus. QM7 cells infected with the indicated PaBV isolates were treated with ribavirin added to the medium in each passage. At regular intervals, subcultures were stained for viral N protein by immunofluorescence and the percentages of positive cells were calculated. (a) Persistently infected cultures were treated for 15 days. (b) Persistently infected cells were treated for 29 days (vertical dotted line) before one subculture was further passaged in the absence of ribavirin. (c) Persistently infected QM7 cells were co-cultured with uninfected QM7 cells at a ratio of 1:20 in the presence or absence of ribavirin. Treatment was stopped after 18 days (dotted line) and the cells were kept in ribavirin-free medium for another 10 days. (a, c) Results for strain PaBV-4 #6758 represent means (±SEM) of two independent experiments.
Fig. 2. Ribavirin reduces activity but not fidelity of the viral polymerase complex. (a) Persistently infected QM7 cells were continuously exposed to 1 µg ml⁻¹ of ribavirin and the indicated concentrations of either guanosine or adenosine. The percentages of viral N protein-positive cells were determined by immunofluorescence at weekly intervals. (b, c, d) QM7 cells persistently infected with PaBV-4 #6758 were treated for 22 days with the indicated concentrations of ribavirin, which was added to the medium at each passage. (b) At regular intervals, subcultures were stained for the viral N protein and the percentages of positive cells were assessed by immunofluorescence. (c) PaBV-4 RNA was quantified by real-time PCR specifically targeting the PaBV-4 P gene (Honkavouri et al., 2008). Viral RNA copy numbers are presented relative to those of the untreated control at each time point. (d) Three fragments of the viral genome were amplified by reverse transcriptase PCR and the mean sequence variation frequency per nucleotide position (3119 positions) was calculated. (e) PaBV-4 polymerase reconstitution assays were performed in chicken DF-1 cells in the absence or presence of the indicated concentrations of ribavirin. At 72 h post transfection, polymerase activity was calculated. Values are presented relative to the untreated control. Results of three independent experiments are shown. The dashed line represents the detection limit of the assay.
of the cytomegalovirus early enhancer/chicken \(\beta\)-actin (CAG) polymerase II promoter. The minigenome carried the Gaussia luciferase gene flanked by the 5' and 3' terminal sequences of the BoDV-1 genome and a hammerhead and a hepatitis delta virus ribozyme sequence (Fig. S2). Firefly luciferase expressed under the control of the CAG promoter was used to normalize for transfection efficiency. Transfected DF-1 cells were left untreated or cultured in medium supplemented with 1, 2 or 5 \(\mu\)g ml\(^{-1}\) of ribavirin for 72 h before luciferase signals were measured and PaBV-4 polymerase activity was calculated (Fig. 2e). Treatment with 1 \(\mu\)g ml\(^{-1}\) of ribavirin reduced polymerase activity to about 10%, while higher concentrations reduced activity to almost background levels (Fig. 2e). Thus, direct interference with viral polymerase activity seems to be the major mode of ribavirin action against avian bornaviruses.

Recent studies demonstrated that ribavirin can enhance type I IFN signalling in mammalian cells, thereby boosting antiviral activity (Liu et al., 2012; Stevenson et al., 2011; Su et al., 2009; Thomas et al., 2011). To investigate whether this effect might also apply to avian cells, we used quail CEC-32 cells expressing firefly luciferase under the control

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**Fig. 3.** Ribavirin and type I IFN have synergistic antiviral effects in avian cells. (a) CEC-32/chMx-Luc reporter cells were either cultured with the indicated doses of ribavirin overnight or stimulated with recombinant chIFN-\(\alpha\) for 6 h before luciferase activities were measured. Luciferase activities are presented as relative light units (RLU). The dashed line represents the mean RLU of untreated controls. (b) CEC-32/chMx-Luc reporter cells were pretreated with the indicated dose of ribavirin overnight and subsequently stimulated with recombinant chIFN-\(\alpha\) (2.5 U ml\(^{-1}\)) for 6 h. Mx reporter-stimulating activity is plotted as fold induction relative to cells stimulated with 2.5 U ml\(^{-1}\) of chIFN-\(\alpha\) alone (dashed line). (c) QM7 cells persistently infected with the indicated PaBV isolates were treated with 1 \(\mu\)g ml\(^{-1}\) of ribavirin, 10 U ml\(^{-1}\) of recombinant chIFN-\(\alpha\) or a combination of both. Fresh compounds were added to the medium at each passage. Subcultures were stained for the viral N protein by immunofluorescence at weekly intervals, and percentages of positive cells were calculated. Cultures were treated for 29 days (dotted line) and subsequently passaged without treatment. Results represent means (\(\pm\)SEM) of four (a, b) or two (c) independent experiments.
of the IFN-inducible chicken Mx gene promoter (CEC-32/chMx-Luc) (Penski et al., 2011). Treatment of these cells with recombinant chIFN-α (Schultz et al., 1995) resulted in a dose-dependent induction of luciferase activity, while slightly enhanced luciferase signals were only observed after treatment with very high doses of ribavirin (Fig. 3a). However, when cells were pretreated with ribavirin before stimulation with a low dose of chIFN-α (2.5 U ml⁻¹), IFN activity was clearly increased up to eightfold as compared to chIFN-α alone (Fig. 3b). This dose-dependent effect of ribavirin suggests a synergism between ribavirin and type I IFN as previously reported for mammalian cells (Thomas et al., 2011).

To assess whether a combined treatment of ribavirin and type I IFN might result in enhanced antiviral activity against avian bornaviruses, we treated persistently PaBV-infected QM7 cultures with either 1 µg ml⁻¹ of ribavirin, 10 U ml⁻¹ of chIFN-α or both. Ribavirin or chIFN-α alone reduced the number of bornavirus-positive cells only mildly (Fig. 3c). In contrast, a combination of both compounds resulted in markedly more pronounced effects with only few virus-positive cells remaining detectable in the cultures after 4 weeks of treatment. When treatment was stopped at day 29 post infection, the proportions of virus-positive cells increased quickly (Fig. 3c), indicating that the combined treatment only repressed rather than eliminated the virus.

Our results confirmed that, like the mammalian bornavirus BoDV-1 (Jordan et al., 1999; Lee et al., 2008; Mizutani et al., 1998, 1999), parrot bornaviruses are susceptible to ribavirin treatment in cell culture. Interestingly, we found that continuous ribavirin treatment of infected avian QM7 cell cultures resulted in a marked decrease of viral RNA levels and bornavirus-positive cells as assessed by indirect immunofluorescence, but complete virus elimination was not achieved, even when ribavirin was combined with IFN-α. At present we cannot distinguish between the possibilities that (i) ribavirin successfully eliminated the virus from the vast majority of cells in our cultures followed by de novo infection after the drug is removed and (ii) ribavirin simply repressed viral activity in the infected cells leading to reduced detectability by immunofluorescence staining without actually eliminating the virus from individual cells.

The marked antiviral effect makes ribavirin a promising candidate for the treatment of bornavirus-infected birds despite its apparent inability to completely eliminate the virus from infected cell cultures. A reduction of viral loads in the organism of a treated bird may decrease viral shedding, and thereby minimize the risk of bornavirus transmission. Furthermore, it may enable the bird’s immune system to eliminate or permanently control the persistent infection. Hoppes et al. (2013) reported that peroral application of ribavirin at 18 µg (kg bodyweight)⁻¹·twice daily had no effect on bornavirus shedding of naturally infected African gray parrots, suggesting that higher doses might be required. However, higher ribavirin doses are not well tolerated by psittacines (M. Rinder, unpublished results). Results from our current cell culture study indicate that the antiviral activity of ribavirin may be greatly enhanced when used together with type I IFN. Thus, in future in vivo experiments, ribavirin should be combined with type I IFNs in order to enhance its effectiveness in psittacines. Unfortunately, the IFN system of parrots is not well characterized. It is unknown if chIFN-α is sufficiently cross-reactive in these species and homologous type I IFN is currently not available for therapeutic approaches.

To elucidate the mechanism by which ribavirin inhibits avian bornaviruses, we evaluated three alternative modes of action that had previously been noted for other viruses. Our results did not allow any firm conclusions regarding the role of drug-induced reduction of the cellular GTP pool, but we found no indication that ribavirin markedly reduces the fidelity of the PaBV-4 polymerase. The PaBV-4 polymerase was highly sensitive to inhibition by ribavirin in a polymerase reconstitution assay, suggesting that the drug directly inhibits the viral polymerase, as previously shown for other RNA viruses (Arias et al., 2008; Hofmann et al., 2008; Parker, 2005; Pfeiffer & Kirkegaard, 2005; Te et al., 2007; Young et al., 2003).

In summary, this study demonstrates that ribavirin inhibits the polymerase of avian bornaviruses and thereby has a potent antiviral effect against these viruses in cell culture. Its efficiency can be further increased by synergistic effects with recombinant IFN-α in avian cells. Our results suggest that a combination of ribavirin and type I IFNs may help to overcome the shortcomings of ribavirin treatment of psittacines in vivo.

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