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Short Communication

Depletion of GTP pool is not the predominant mechanism by which ribavirin exerts its antiviral effect on Lassa virus

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ABSTRACT

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is the standard treatment for Lassa fever, though its mode of action is unknown. One possibility is depletion of the intracellular GTP pool via inhibition of the cellular enzyme inosine monophosphate dehydrogenase (IMPDH). This study compared the anti-arenaviral effect of ribavirin with that of two other IMPDH inhibitors, mycophenolic acid (MPA) and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR). All three compounds were able to inhibit Lassa virus replication by ⩾2 log units in cell culture. Restoring the intracellular GTP pool by exogenous addition of guanosine reversed the inhibitory effects of MPA and EICAR, while ribavirin remained fully active. Analogous experiments performed with Zaire Ebola virus showed that IMPDH inhibitors are also active against this virus, although to a lesser extent than against Lassa virus. In conclusion, the experiments with MPA and EICAR indicate that replication of Lassa and Ebola virus is sensitive to depletion of the GTP pool mediated via inhibition of IMPDH. However, this is not the predominant mechanism by which ribavirin exerts its in-vitro antiviral effect on Lassa virus.

Lassa virus is a segmented negative strand RNA virus of the family *Arenaviridae*. The virus causes Lassa fever, a hemorrhagic fever endemic in West Africa. The case fatality rate in hospitals is about 20% (McCormick et al., 1987). The outcome can be significantly improved by administration of ribavirin, in particular if the drug is given early (McCormick et al., 1986). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) in its 5′-monophosphorylated form is an inhibitor of the cellular enzyme inosine monophosphate dehydrogenase (IMPDH) (Streeter et al., 1973) and displays broad-spectrum antiviral activity against RNA viruses in vitro (Sidwell et al., 1972). Several mechanisms have been proposed by which the compound may inhibit the replication of various viruses (i) inhibition of capping of viral mRNA (Goswami et al., 1979) (ii) incorporation into the virus genome by the viral RNA-dependent RNA polymerase (RdRp) leading to lethal mutagenesis, also called error catastrophe (Crotty et al., 2000) (iii) inhibition of the viral RdRp by the 5′-triphosphate (Eriksson et al., 1977; Fernandez-Larsson et al., 1989) and (iv) depletion of intracellular GTP pool via inhibition of IMPDH (Leyssen et al., 2005; Smee et al., 2001). Currently, there are no experimental systems available to test whether ribavirin 5′-triphosphate directly targets the RdRp of Lassa virus. Ribavirin, at high concentrations, was shown to inhibit the Lassa-related Old World arenavirus lymphocytic choriomeningitis virus (LCMV) by a mechanism unrelated to lethal mutagenesis (Ruiz-Jarabo et al., 2003). To obtain insight into the mechanism of action, we studied whether depletion of intracellular GTP pool by ribavirin is responsible for inhibition of Lassa virus replication. The antiviral effect of ribavirin was compared with that of two other IMPDH inhibitors known to inhibit viral replication, mycophenolic acid (MPA) (Ando et al., 1968; Franklin and Cook, 1969) and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) (Balzarini et al., 1993; De Clercq et al., 1991). To test for the relevance of IMPDH inhibition, the antiviral activity of the compounds was measured while simultaneously the intracellular GTP level was restored by exogenous addition of guanosine. The experiments were conducted with Lassa virus; the closely related African arenavirus Mopeia was used in confirmatory experiments. The filovirus Ebola, which is barely inhibited by ribavirin, served as a control.

Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with streptomycin/penicillin and 10% fetal calf serum. One day before infection, cells were seeded at a density of

Abbreviations: EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; IMPDH, inosine monophosphate dehydrogenase; LCMV, lymphocytic choriomeningitis virus; MOI, multiplicity of infection; MPA, mycophenolic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide; RdRp, RNA-dependent RNA polymerase; Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide.

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4×10^4 cells per well of a 24-well plate. Cells were inoculated with Lassa virus strain AV or Mopeia virus strain 21366 at a multiplicity of infection (MOI) of 0.01 or with Zaire Ebola virus at an MOI of 0.01. After 1 h, cells were washed with phosphate-buffered saline and fresh medium with or without compound and different concentrations of guanosine was added. Infections with Lassa and Ebola virus were carried out under biosafety level four conditions. Concentration in cell culture supernatant of genome copies and infectious virus particles was measured using quantitative real-time RT-PCR and immunofocus assay, respectively, 48 h p.i. (Lassa and Mopeia virus) or 72 h p.i (Zaire Ebola virus), i.e. when the end of the exponential growth phase has been reached (Günther et al., 2004). Quantitative RT-PCR was performed as described (Panning et al., 2007), and by using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) in combination with Lassa virus primers 36E2 and LVS-339-rev (Ölschläger et al., 2010) or Mopeia virus primers MopGPC-fwd (GGTGCCACACATCCTTGAAGA) and MopGPC-rev (GGCA TCGTTGCATTCAGTGA). Infected cell foci in immunofocus assay (Günther et al., 2004) were detected by using Lassa and Mopeia virus NP-specific monoclonal antibodies or Ebola virus-specific goat antibodies. Titers were expressed as focus-forming units/ml. Cell growth and viability under compound treatment was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) method as described (Günther et al., 2004). Due to the exponential nature of the measurement system (virus growth), data of individual experiments were combined using geometric mean and presented on a log scale. A sigmoidal dose–response curve was fitted to the data using Prism GraphPad 4.0 (GraphPad Software). IC_{50} and IC_{90} values were calculated from the sigmoidal functions.

The effect of ribavirin, EICAR, and MPA on the release of virus genome-containing particles and infectious units is shown in Figs. 1 and 2, respectively. The graphs in Fig. 2 represent the average of four independent infection experiments performed successively. Due to variability in exponential virus growth, spanning up to 4 log units during the assay period (Günther et al., 2004), and differences in the inhibition kinetics between individual cell culture experiments, the readout data were more widely scattered over the effective concentration range of the compounds. The error bars in Fig. 2 represent minimum and maximum to demonstrate the full data range.

All three compounds were able to inhibit Lassa virus replication by about 2 log units, irrespective of whether genome copies (Fig. 1) or infectious particles (Fig. 2, top row) were measured. However, the compounds have a different inhibition profile with EICAR and MPA being more active at lower concentrations, while the inhibitory effect of ribavirin occurs more abruptly at relatively high concentrations. The antiviral effect of ribavirin against Lassa virus was not reduced when guanosine was added to the culture medium, even not at a concentration of 30 μg/ml (Fig. 2). The mean IC_{50} and IC_{90} values were essentially unchanged (Table 1) and the virus titer was below the detection limit in all experiments with 100 μg/ml ribavirin, irrespective of the guanosine concentration. In contrast, the inhibitory effect of MPA on Lassa virus was efficiently reversed in the presence of low concentrations of exogenously added guanosine. The effect of EICAR was also reversed at 3 μg/ml guanosine. Paradoxically, at 30 μg/ml guanosine some inhibitory effect re-appeared. This effect was observed in all four repeat experiments, although the degree of inhibition varied. The underlying pharmacological or metabolic changes of this phenomenon are unclear. The MTT test indicated only minor growth inhibition in the test range of the compounds and at all guanosine concentrations used (Fig. 3A). The experiments with ribavirin and MPA were repeated with Mopeia virus, which confirmed the results obtained with Lassa virus (Table 1). These data suggest that ribavirin does not exert its antiviral effect on Lassa and Mopeia virus via GTP pool depletion. In contrast, the inhibitory effects of EICAR and MPA are likely to originate predominantly, if not exclusively, from depletion of the GTP pool. Since ribavirin and MPA appear to inhibit arenavirus replication through different mechanisms, we wondered if they could act in an additive way. To this end, both compounds were tested in various combinations on Mopeia virus-infected cells (Fig. 3B). Additive effects were observed in the concentration ranges of 0.03–0.1 μg/ml MPA and 1–3 μg/ml ribavirin; at these concentrations, an additional treatment with the other compound clearly intensified inhibition. At higher concentrations the plateau was reached.

Lassa virus produces in cell culture an excess of about 100 genome-containing particles per infectious unit as measured in an immunofocus assay (Asper et al., 2004). To test if ribavirin treatment changes this ratio, cells were infected with Lassa or Mopeia virus and the inhibition kinetics were determined in parallel for virus genomes and infectious units (Fig. 3C). For both viruses, the release of infectious particles was more strongly inhibited than that of virus genomes, suggesting that ribavirin treatment increases the relative fraction of defective virus particles.

The effects of the compounds on Zaire Ebola virus were less pronounced, with an inhibition hardly reaching 1 log unit (Fig. 1 and 2, top row). The effects of EICAR and MPA were reversed by addition of guanosine (Fig. 2 and Table 1), indicating that GTP pool depletion is the predominant mechanism of action of these two compounds against Ebola virus. No clear conclusions as to the role of GTP pool depletion could be drawn from the experiments with ribavirin, as the addition of guanosine only had minor effects, if at all.

In conclusion, the inhibition of Lassa virus by MPA and EICAR and the reversal of the antiviral effect by addition of guanosine demonstrate that replication of arenaviruses is sensitive to depletion of the GTP pool via inhibition of IMPDH. However, ribavirin does not seem to act primarily via depletion of GTP pool against Lassa virus. Similar data have been obtained for Hantaan virus (*Bunyaviridae*) (Sun et al., 2007), and there is evidence that ribavirin causes an error catastrophe during Hantaan virus replication (Severson et al., 2003). On the contrary, the activity of ribavirin against yellow fever virus (*Flaviviridae*) and human parainfluenza virus (*Paramyxoviridae*) has been linked to inhibition of IMPDH; inhibitory effects in Vero cells could be efficiently reversed by addition of 10 μg/ml guanosine (Leyssen et al., 2005). No evidence for an enhanced error rate was found with yellow fever virus (Leyssen et al., 2006). The inhibitory effect of ribavirin on Lassa and Mopeia virus appears to be associated with a preferential release of defective particles relative to infectious particles. The ratio between RNA copies and infectious units increased 10-fold when ribavirin was added in concentrations exceeding the IC_{90}. Whether

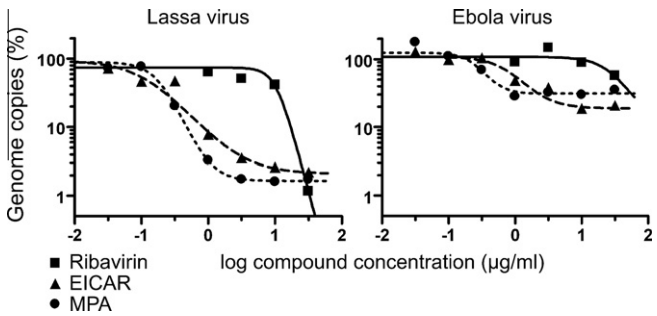

Fig. 1. Inhibition of replication of Lassa virus and Zaire Ebola virus by ribavirin, EICAR, and MPA in vitro. Concentration of virus genome copies in cell culture supernatant was measured by quantitative real-time RT-PCR. Two to four controls without treatment were included to determine the 100% value. Geometric means of data from two cell culture experiments are shown.

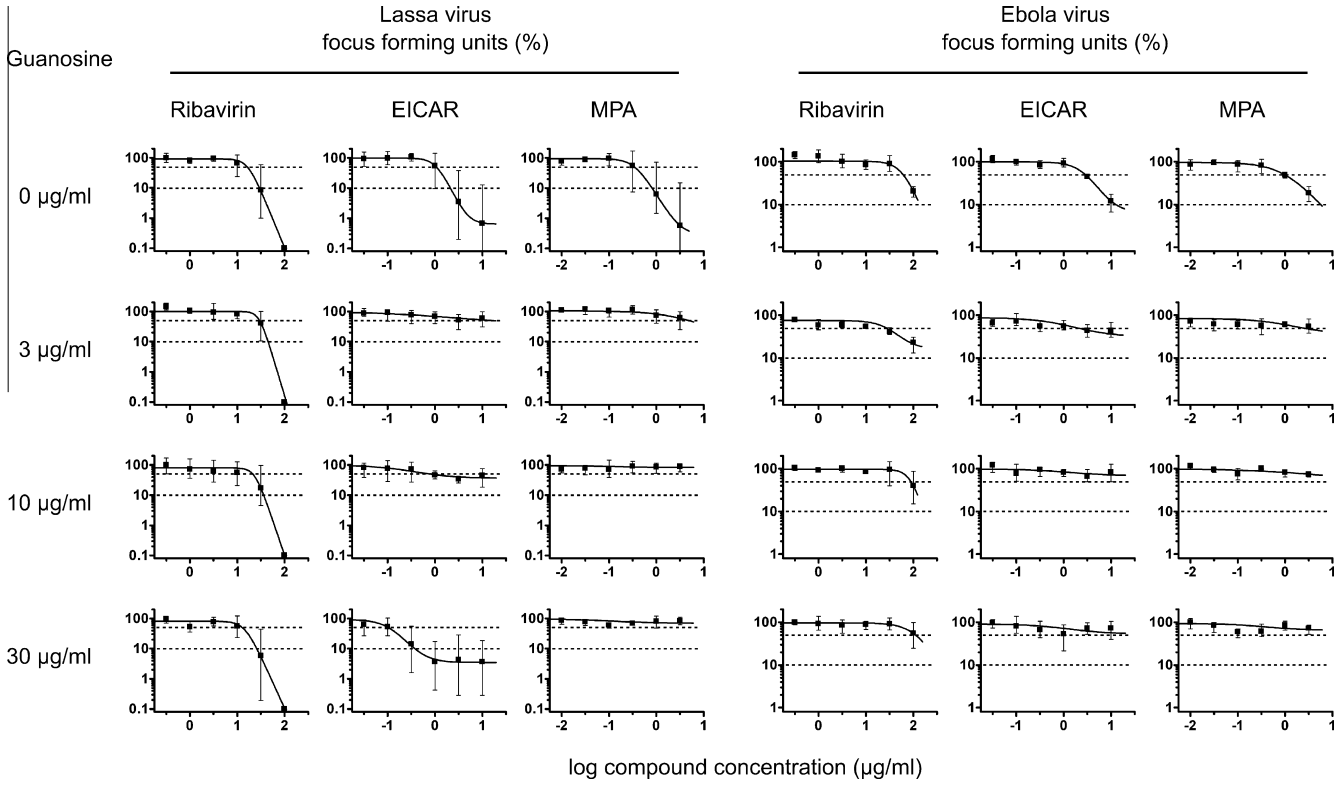

Fig. 2. Effect of ribavirin, EICAR, and MPA on replication of Lassa virus and Zaire Ebola virus in vitro depending on the concentration of exogenously added guanosine. Concentration of infectious virus particles in cell culture supernatant was measured by immunofocus assay. Geometric means of four (Lassa virus) and three (Ebola virus) independent cell culture experiments are shown. Error bars represent minimum and maximum of the data. Each experiment included six controls without treatment to determine the 100% value. The concentration of guanosine is shown on the left of the respective row. IC_{50} and IC_{90} levels are indicated by dotted lines. The corresponding IC_{50} and IC_{90} values are shown in Table 1. No focus-forming units of Lassa virus were detected in any experiment under treatment with 100 µg ribavirin per ml, irrespective of the concentration of guanosine. These values are shown at the limit of detection of the immunofocus assay.

Table 1
Inhibition of replication of Lassa virus, Mopeia virus, and Zaire Ebola virus in vitro by ribavirin, EICAR, and MPA in the presence or absence of exogenously added guanosine[a]

Virus (family)	Guanosine (µg/ml)	Ribavirin (µg/ml)		EICAR (µg/ml)		MPA (µg/ml)	
		IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Lassa (arena)	0	16	29	1.0	2.1	0.34	0.81
	3	29	44	>10	>10	>3	>3
	10	21	36	0.79	>10	>3	>3
	30	13	26	0.10	0.38	>3	>3
Mopeia (arena)	0	5.4	10	ND	ND	0.17	0.34
	3	2.6	6.2	ND	ND	>3	>3
	10	2.2	6.0	ND	ND	>3	>3
	30	2.5	8.1	ND	ND	>3	>3
Ebola (filo)	0	61	>100	2.8	>10	0.96	>3
	3	27	>100	2.0	>10	2.5	>3
	10	89	>100	>10	>10	>3	>3
	30	>100	>100	>10	>10	>3	>3

ND: not done.
[a] All values are based on measurement of infectious virus particles.

or not our data indicate that ribavirin is incorporated into virus genomes leading to an error catastrophe with increased production of non-viable virus particles is a matter of further investigation. Previous experiments with LCMV indicated that ribavirin does not enhance the mutation rate of arenaviruses (Ruiz-Jarabo et al., 2003). An increase in the relative fraction of defective viruses could be equally well explained by budding of immature particles lacking glycoproteins, L protein, or L RNA segments (the RT-PCRs used in our study targeted the S RNA segment) due to shortage of or imbalance between these essential virion components in ribavirin-treated cells. Alternative hypotheses for the mode of action of ribavirin include competitive or allosteric binding to the Lassa virus RdRp as suggested for influenza virus (Eriksson et al., 1977) or premature termination of elongation following incorporation into the nascent RNA strand. As ribavirin is a guanosine analogue and Lassa virus generates its mRNAs by cap-snatching (Lelke et al., 2010; Morin et al., 2010), the drug may also interfere with binding of the capped primer to the ribonucleoprotein complex.

The ribavirin experiments with Zaire Ebola virus were inconclusive, as the inhibitory effect was small – as expected – and addition of guanosine was not associated with clear changes. However, the experiments with EICAR and MPA indicate that Ebola virus

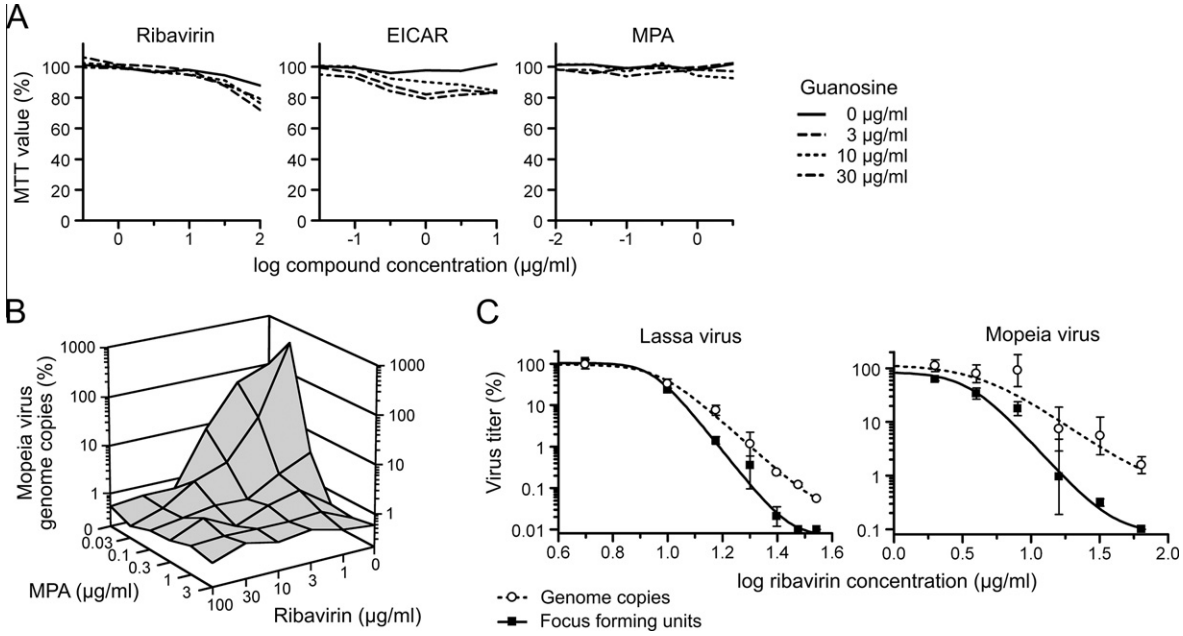

Fig. 3. (A) Influence of ribavirin, EICAR, MPA, and guanosine on cell growth and viability. Vero cells were incubated with the compounds for 48 h and subsequently assayed by MTT test. (B) Effect of ribavirin–MPA combination treatment on Mopeia virus replication. Vero cells were infected with Mopeia virus and the concentration of virus genome copies in cell culture supernatant was measured by quantitative real-time RT-PCR. Various concentrations of both compounds were tested in a 6×6 matrix, resulting in 36 data points represented by the intersection points of the grid lines on the 2-dimensional surface plot. Geometric means of triplicates are shown. (C) Selective influence of ribavirin treatment on release of genome-containing virus particles and infectious units. Vero cells were infected with Lassa virus (in triplicate) or Mopeia virus (in duplicate) and the concentrations of virus genome copies and infectious units in cell culture supernatant were determined in parallel by quantitative real-time RT-PCR and immunofocus assay, respectively. Geometric means of replicates are shown. Error bars represent minimum and maximum of the data.

replication can be inhibited to some extent by depletion of the of the GTP pool, an observation which may have implications for drug development and usage.

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