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PRINCIPAL INVESTIGATOR: Jean L. Patterson

CONTRACTING ORGANIZATION: Children's Hospital Corporation
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Boston, MA 02115

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Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) or Virazole is a broad-spectrum antiviral agent whose molecular mode of action remains remarkably controversial. The drug was approved by the Food and Drug Admistration in 1986 for aerosol use in infants with serious infections due to respiratory syncytial virus (RS). Ribavirin is and has been under clinical investigation against a variety of viral illness, including those due to influenza virus, Lassa fever, Korean hemorrhagic fever with renal syndrome (KHFS) and Human immunodeficiency virus (HIV). There has been a great deal of clinical interest in utilizing ribavirin for HIV infections. It has been reported to slow the development of AIDS in HIV infected patients (McCormick et al., 1984). We describe here the reported mechanisms of action of this newly licensed drug.

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Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) or Virazole is a broad-spectrum antiviral agent whose molecular mode of action remains remarkably controversial. The drug was approved by the Food and Drug Administration in 1986 for aerosol use in infants with serious infections due to respiratory syncytial virus (RS). Ribavirin is and has been under clinical investigation against a variety of viral illness, including those due to influenza virus, Lassa fever, Korean hemorrhagic fever with renal syndrome (KHFS) and Human immunodeficiency virus (HIV). The drug possesses inhibitory activity against a broad spectrum of viral pathogens, including both DNA and RNA viruses (Sidwell et al,.1972), intrinsically suggesting a vast clinical potential that has yet to be realized.

There has been a great deal of clinical interest in utilizing ribavirin for HIV infections. It has been reported to slow the development of AIDS in HIV infected patients (McCormick et al., 1984). The mechanism for the interference of ribavirin on HIV is as yet not understood. Recently Vogt et al (1987) reported that this drug was antagonistic with AZT in HIV infected cells. They hypothesized that since both drugs work in some part as a triphosphate that there is competition for cellular kinases.

Several theories regarding the molecular mode of action of ribavirin have been proposed. One hypothesis states that the drug leads to decreased intracellular pools of GTP,

indirectly suppressing viral nucleic acid synthesis (Malinoski and Stollar, 1981 and Streeter et al., 1973). Another hypothesis proposes that ribavirin therapy of viral-infected cells results in the synthesis of RNA with abnormal or absent 5′ cap structures, which in turn leads to inefficient translation of viral transcripts (Goswami et al., 1979). A third hypothesis states that the drug has a direct suppressive effect on viral polymerase activities. Experimentally it has been difficult to determine the primary mechanism of action because none of these hypotheses are mutually exclusive, and indeed, they may indicate that ribavirin acts in a complex, multiple site fashion. It is also important to note that ribavirin, unlike all other drugs whose structures resemble nucleoside analogues, has a modified base. Antiviral agents, notably AZT, have modified sugars and generally are terminators of growing nucleic acid chains. Their major mode of action was relatively simple to determine.

Ribavirin was initially described as closely resembling guanosine in structure (Prusiner and Sundaralingam, 1973) and it was found that when cells were treated with this drug pools of GTP were diminished (Smith, 1984). Early after the development of this drug, ribavirin-5′-monophosphate was shown to be a potent inhibitor of IMP dehydrogenase activity (Malinoski and Stollar, 1981). The hypothesis was made that the remaining cellular and viral enzymes were thought to compete for the depleted concentration of GTP. The major

evidence against this being the primary mode of antiviral action is that other compounds notably, the 1,4,5-triazole derivative of ribavirin which deplete GTP pools, have no antiviral activity (Smith, 1984). Again, it is important to note that no mechanism excludes another, all could work and thereby enhance each other's effectiveness.

The hypothesis that ribavirin exerts its antiviral effect by blocking capping comes from early work with vaccinia virus. Katz et al (1976) reported that ribavirin inhibited vaccinia virus growth in BSC I cells. Fifty ug/ml of virazole inhibited virus growth more than ten-fold, the effect, however, was not linear, because 25 ug/ml caused only a two-fold decrease. Equimolar concentrations of guanosine added to cells resulted in loss of the inhibitory effect. In an assay measuring the appearance of viral particles ribavirin interfered dramatically. Their final conclusion was that ribavirin interfered with DNA synthesis in some unknown fashion. More recently Goswami et al (1982) have used purified vaccinia virus capping enzymes and shown that ribavirin 5'-triphosphate was an effective inhibitor of the viral mRNA (guanine-7) methyltransferase, indeed by their kinetic analysis it appeared to be a direct competitive inhibitor. Canonico et al (1980) reported that when examining mRNA from Eastern equine encephalitis virus infected cells that the transcripts appeared to have lost their cap structure. In another reported set of experiments

(Toltzis and Huang, 1986) in which the effects of ribavirin on vesicular stomatitis virus (VSV) grown in Chinese hamster ovary cells were examined, it was indicated that the drug leads to the synthesis of inefficiently translated viral mRNA. There was only a little demonstrable effect on viral primary transcription, lending support to the hypothesis that the drug alters RNA cap structures.

The problem with these and other experiments dealing with translational efficiencies and capping is that it is not clear that a cap is an absolute requirement for translation. Polio virus which does not have a cap on its mRNA translates adequately. Ironically, we have learned a great deal about the function, or lack thereof, of cap structures from this virus (see Ehrenfeld, 1982). More importantly, <u>in vitro</u> translation of mRNAs is notoriously unfaithful to the situation inside the infected cell. Rabbit reticulocyte lysate, in particular, will initiate internally on a message producing truncated proteins where none exist inside the cell. Unfortunately, the assay to accurately measure translational efficiency is yet to be developed.

The idea that ribavirin may directly affect polymerases has been reported previously for other viruses, notably influenza virus (Wray et al., 1985). Work in our lab has indicated that the structural similarity of ribavirin may include all of the natural nucleoside triphosphates.

Results of our experiments in which the effects of phosphorylated ribavirin compounds on an in vitro VSV polymerase assay were examined, indicated that the drug does indeed possess a significant direct suppressive effect on viral polymerase (Toltzis et al., 1988). In fact all three phosphorylated species inhibited VSV transcription. The mono- and diphosphorylated forms of the drug possessed approximately two to three times the inhibitory activity as the triphosphorylated form. Transcripts synthesized in the presence of the drug were full-length. Inhibition by ribavirin 5'-diphosphate could be reversed by the addition of UTP, CTP and GTP. While the addition of GDP to the reaction did not reverse inhibition. Nearest neighbor analysis supported the notion that none of the phosphorylated forms were incorporated into the growing chain of RNA. Surprisingly, in contrast to the viral mRNA synthesized in infected cells in the presence of ribavirin transcripts synthesized in vitro (in the test tube) in the presence 5' mono-, di-, and triphosphorylated forms of the drug translated with equal efficiencies under the test conditions. This results either suggest a contradiction to the theory that ribavirin affects cap structures and interferes with translation, or that the in vitro RNA synthesis system cannot mimic the cellular machinery accurately.

Our initial observation that besides the triphosphorylated form both the mono- and di-phosphorylated

forms of the drug were active against the VSV replicase was surprising. It has been shown previously that all nucleoside analogs inhibit viral polymerases in the triphosphorylated form (Eriksson et al., 1977, Furman et al., 1984). We have used enzyme kinetic procedures and product analysis to further investigate the mechanism of action of ribavirin. When analyzed by double-reciprocal plots both RDP and RTP gave similar patterns of inhibition, although the Km values of the nucleoside triphosphates in the presence of the different drugs are not the same (Fernandez-Larsson et al., 1989). Both of the phosphorylated forms of ribavirin compete directly with all four of the nucleoside triphosphates, suggesting that they are acting on the polymerase in a similar fashion. Because there appears to be no incorporation of ribavirin into the growing nucleic acid chain and because the transcripts synthesized appear full length in the presence of both drugs it was our initial hypothesis that RDP and RTP were blocking polymerase initiation at the 3′ terminus of the genome.

We then studied purified <u>in vitro</u> transcription products synthesized in the presence and absence of phosphorylated ribavirin compounds to determine the effect of these drugs on leader RNA and mRNA synthesis and to further characterize the observations on enzyme kinetics. VSV leader RNA is a 47 nucleotide noncapped and nonpolyadenylated RNA transcript which is the first RNA synthesized after the polymerase begins transcription, it is complimentary to the exact 3′

end of the negative stranded RNA genome. The next transcript synthesized is the nucleocapsid (N) mRNA. It is both capped and polyadenylated. We found that the inhibition of leader synthesis was similar to the inhibition of N mRNA synthesis, since the ratios of these two RNA transcripts did not appear to change with the addition of RDP or RTP to the transcription reaction. The results suggest that the inhibitory effect of ribavirin on the polymerase occurs at the level of initiation of primary transcription, when the transcriptase enters the 3' end of the genome. Later, using the same type of analysis we were able to show that a VSV mutant had an altered ATP function. Kinetic analysis clearly showed that the direct competitive effect of RDP or RTP was different in this mutant (Fernandez-Larsson and Patterson, 1989). The altered ATP function observed in the kinetic analysis is compatible with the argument for an additional ATP-binding site on the polymerase complex. In this mutant RTP and RDP seemed to interact in a different manner with the ATP binding sites than they do with wild-type VSV.

In another viral system, reovirus, researchers reported that 12.5 uM of ribavirin inhibits all stages of viral replication (Rankin et al., 1989). Reovirus is a dsRNA virus. When RTP was tested on an <u>in vitro</u> transcription reaction utilizing the dsRNA genomes as template, both elongation and initiation of plus stranded mRNAs were inhibited. There was no effect on cap formation and/or

methylation. These results are quite similar to the VSV situation where no effect on apparent in vitro translatability could be found when mRNA was synthesized in vitro. These authors have suggested a model that supports RTP binding to a catalytic site on the transcriptase, again similar to what we have seen with VSV.

Our data and others have suggested that ribavirin has an effect on the initial steps of transcription by RNA-dependent RNA polymerases and that this effect may be mediated by several phosphorylated forms. One of the reasons that the antiviral activity of ribavirin maybe so formidable to pin down is that the inhibition of polymerases, without chain termination, is more difficult to assess. However, if indeed primary transcription is affected, direct linear effects of the drug on later stages in viral replication and assembly are difficult to ascertain as any deregulation of the system at the mRNA level is presumably amplified at all levels of viral progeny production. These small effects on primary transcription, may be enough to produce the entire antiviral activity seen, with respect to diminished viral titres. Unfortunately there is little information on how ribavirin may interfere with DNA viruses.

All antiviral compounds must be evaluated recognizing that by the time "self-limiting" diseases are recognized

most replication and viral progeny production is accomplished. The damage is done, so to speak. It is not fair to compare these compounds with antibacteriocidal agents. The reality is that most of the effective antivirals will only work on chronic infections. Nevertheless, as the development of antiviral compounds grows, we need to adequately prepare ourselves to determine, primarily via rapid viral diagnostics, which virus is the agent and the length of the viral infection.

Ribavirin remains an important potential agent in the treatment of a broad spectrum of serious viral illnesses encountered worldwide and may serve as a model for similar, more effective agents in the future. Definition of its molecular activity will be vital to the development of its use and the use of similar agents.

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