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THOMAS STAPLETON

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Introduction

THOMAS STAPLETON

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I would like to welcome you to this very important meeting here today. It is not very often that what looks like a really new and valuable chemical for treating a very common and serious paediatric disease occurs, and I think we are all going to be very interested to learn more about ribavirin.

Professor John Davis who is Foundation Professor of Paediatrics at the University of Cambridge and for some time was Chairman of the Academic Board of the British Paediatric Association is going to moderate this morning's meeting, and introduce the speakers.

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Ribavirin: a broad spectrum antiviral agent

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Until a couple of decades ago it was believed that viral chemotherapeutic agents would never be found. According to the widely held view, viruses replicated only by taking over the host cell's metabolic machinery. Thus any drug capable of inhibiting viral replication would do so only through non-specific cytotoxicity and would obviously be too toxic for clinical use. During the past 20 years this very discouraging view has, I believe, been laid to rest. It is clear that viruses replicate by processes other than simple appropriation of standard cells, demonstrated by *in vitro* experimental animal and clinical findings, yet the issue of cytotoxicity is still a very large challenge. Selectivity for viral replication can be enhanced by targeting agents to viral specific processes. Whether this involves viral specific enzymes or viral specific events (such as absorption, penetration, uncoating or assembly) or specific host cell processes that are vastly enhanced during viral replication such as the replication of genetic material, the elaboration and processing messenger RNA or the elaboration and processing viral specific proteins, is as yet unclear.

Over the years chemists have provided us with a number of substances with varying levels of toxicity which selectively inhibit viral replication. Several recent reviews cover these agents and a particularly excellent recent one was authored by Professor R.K. Robins (1). Of all the viral chemotherapeutic agents thus far developed ribavirin stands alone in demonstrating antiviral activity against a broad spectrum of both RNA and DNA viruses.

To the casual observer, ribavirin may appear more as a pyrimidine analogue than any other structure. But single crystal X-ray diffraction analysis (2) clearly established its close resemblance in structure to guanosine (Fig. 1). The geometry about the carboxamide function is the key to this similarity. The carbonyl oxygen and the amide nitrogen at position 7 and 8 respectively of ribavirin occupy similar positions stereochemically to the carbonyl oxygen and the ring nitrogen in position 10 and 1 of guanosine. This structural similarity has been a guiding principle in studies on the mode of action of ribavirin suggesting that guanosine nucleotides may in some way be involved.

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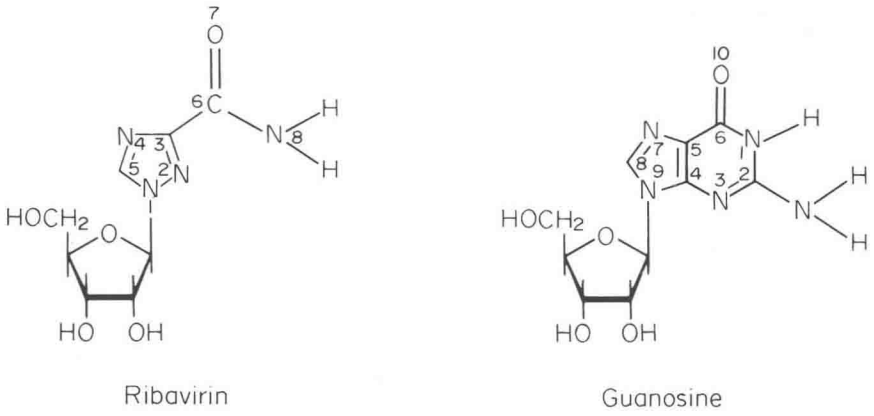

Figure 1. Structures of ribavirin and guanosine.

The view that the mechanism of action of ribavirin must involve guanosine nucleotides was strengthened considerably by experiments carried out at ICN (3) in which the reversal of the anti-viral effect of ribavirin was shown when guanosine was added to Vero cell line infected with measles virus (Table 1). In addition to guanosine, xanthosine effectively reversed the antiviral activity but inosine was somewhat less effective.

Table 1

Effect of nucleotides and nucleosides on the anti-measles virus activity of ribavirin in vero cell culture[a]

	Total plaque-forming units of measles infected vero cells *vs* conc. of (μg/ml) ribavirin and various nucleosides.			
Compound	500	100	20	0
Ribavirin	0	3	176	213
Ribavirin + guanosine	29	168	196	202
Ribavirin + xanthosine	53	211	215	215
Ribavirin + inosine	0	125	166	177
Ribavirin + orotidine	0	16	191	210

Adenosine, deoxyadenosine, cytidine, uridine, thymidine, Aicar, Aicar-5′-P = no reversal.

[a]200 μg/ml of each nucleotide or nucleoside were incorporated in the agar overlay with each concentration of ribavirin. The agar overlay was added onto the cultures after 1–1·5 h of virus adsorption.

Orotidine, adenosine, deoxyadenosine, and aminoimidazolecarboxamide ribonucleotide were not effective in reversing the virustatic effect. Similar results have been obtained in other laboratories, showing that guanosine abolished the cytostastic effect of ribavirin on non-infected mouse lymphoma cells (L5178Y) (4) and also reversed the inhibition of haemagglutinin production by influenza A virus in MDCK cells (5). Nonetheless, guanosine does not reverse the antiviral effect of ribavirin in mice infected with influenza virus (6). On balance, both chemical and biological evidence suggests that ribavirin simulates guanosine in structure and thus its principle mode of action might be expected to involve guanosine nucleotides.

Early *in vitro* testing against a broad spectrum of viruses showed that ribavirin was active against both DNA and RNA viruses (8). Ribavirin clearly showed significant activity against bacteriophages in prokaryotic cells (9) and activity against viral infections of plant and animal cells had been reported (9,10). Inspection of Tables 2 and 3 reveals that the range of ribavirin antiviral activity is indeed wide. Unlike other antivirals, development of ribavirin resistant virus strains has not been demonstrated. In most resting cell lines in which antiviral evaluations are performed, the antiviral activity can usually be separated from any cytostatic dose, which ranges from 200 to 1000 μg of ribavirin per ml. The minimum virus inhibitory concentrations, on the other hand, range as low as 0.001 μg/ml. Such observations suggest that there is a considerable selectivity of antiviral effect in the case of the more sensitive viruses.

Table 2

DNA viruses reported to be inhibited in vitro *by ribavirin*[a]

Virus	References
Adeno 3	Sidwell *et al.* (1972); Hoffman *et al.* (1973)
Adeno 19	Scheffler *et al.* (1975)
Herpes 1	Sidwell *et al.* (1972); Hoffman *et al.* (1973); Descamps and De Clercq (1978)
Herpes 2	Sidwell *et al.* (1973); Hoffman *et al.* (1973); Descamps and De Clercq (1978)
Turkey herpes	C. Eidson, cited by Sidwell *et al.* (1974)
Mareks disease	Eidson *et al.* (1974)
Human cytomegalo	Sidwell *et al.* (1974)
Murine cytomegalo	Sidwell *et al.* (1972); Hoffman *et al.* (1973); Dowling *et al.* (1976)
Feline rhinotracheitis	Povey (1978)
Infectious bovine rhinotracheitis	Hoffman *et al.* (1973)
Vaccinia	Sidwell *et al.* (1972); Hoffman *et al.* (1973) Katz *et al.* (1976); Descamps and De Clercq (1978)
Myoxma	Sidwell *et al.* (1972); Hoffman *et al.* (1973)

[a]From Sidwell (7)

In part the *in vitro* selectivity is dependent on the cell line which is used to assay for antiviral activity (11). The Vero cell line is particularly refractory and requires ribavirin concentrations which are 10 to 100 times greater than required by more sensitive cell lines (7).

It is difficult to account for differences in cell line sensitivity since the uptake of ribavirin by both sensitive and insensitive cells is quite similar (11). It has been shown that 90–95% of intracelluar ribavirin is in the cytoplasmic fraction while only 5–10% become sequestered in lysozymes (11). Ribavirin has proved efficacious in the treatment of animals infected with DNA or RNA viruses. There is a large volume of literature which exists on the subject and which is summarized in Tables 4 and 5.

While therapeutic indices for the influenza and parainfluenza viruses in many animal systems vary in range from 1 to 16, they vary even more widely for certain DNA viruses, such as herpes. In some cases, depending on the model, the

Table 3

Ribavirin RNA viruses reported to be inhibited in vitro *by ribavirin*[a]

Viruses	References
Influenza A and/or B	Sidwell *et al.* (1972); Hoffman *et al.* (1973); Togo (1973); Suganuma and Ishida (1973); Oxford (1975); Durr and Lindh (1975); Appleyard and Maber (1975); Tisdale and Bauer (1975, 1977)
Parainfluenza 1 and 3	Sidwell *et al.* (1972); Hoffman *et al.* (1973); Sidwell *et al.* (1975b)
Parainfluenza 2	Povey (1978)
Measles	Hoffman *et al.* (1973); Streeter *et al.* (1973); Descamps and De Clercq (1977)
Subacute sclerosing panencephalitis	Hoffman *et al.* (1973)
Rhino 1A, 2, 8, 13, 56	Sidwell *et al.* (1972); Hoffman *et al.* (1973)
Type 22A Corona	G. Werner, cited by Sidwell *et al.* (1974)
Type A21 Coxsackie	G. Werner, R. Wenham, C. Engle, cited by Sidwell *et al.* (1974)
Type B Coxsackie	G. Werner, C. Engle, cited by Sidwell *et al.* (1974); Descamps and De Clercq (1977)
Type 1 and 2 Polio	Hoffman *et al.* (1973); N. Ishida and C. Engle, cited by Sidwell *et al.* (1974); Descamps and De Clercq (1977)
Sindbis	G. Werner, cited by Sidwell *et al.* (1974); Descamps and De Clercq (1977)
Vesicular stomatitis	Sidwell *et al.* (1972); Hoffman *et al.* (1973); N. Ishida, cited by Sidwell *et al.* (1974)
Semliki Forest	Sidwell *et al.* (1972); Hoffman *et al.* (1974)
Newcastle disease	Hoffman *et al.* (1973); G. Werner, cited by Sidwell *et al.* (1973)
Moloney sarcoma	M. Chirigos, cited by Sidwell *et al.* (1974)
Gross AKR leukaemia	Shannon (1977)
Rota	Schoub and Prozesky (1977)
Chandipura	Odelola (1977)
Chikungunya	Odelola (1977)
Lassa fever	Jahrling *et al.* (1980)
Rift Valley fever	Stephen *et al.* (1980)
Machupo	Stephen *et al.* (1980)
Sandfly fever	Stephen *et al.* (1980)
Hantaan virus	Huggins *et al.* (1986)

[a]Adapted from Sidwell (7)

therapeutic index may range from 0 to 125. Thus, much is yet to be learned about optimizing the treatment regimen with respect to ribavirin.

Therapeutic effects of ribavirin in the treatment of monkeys infected with Lassa fever or machupo fever viruses however, have been very impressive; Jahrling *et al.* (13) reported 60% of the monkeys infected with 10^6 plaque forming units (PFU) of

Table 4

DNA virus infection significantly inhibited in vivo *by ribavirin*[a]

Virus	Animal host	Route of virus inoculation[b]	References
Herpes 1	Rabbit	c.s. (eye)	Sidwell *et al.* (1972, 1973); Shiota *et al.* (1977)
	Hamster	c.s. (eye)	Sidwell *et al.* (1972, 1973)
	Mouse	s.c. (tail)	Sidwell (1977); Sidwell *et al.* (1972)
	Mouse	s.c. (back)	G. Mayer, cited by Sidwell *et al.* (1974)
	Mouse	i.n.	De Clercq *et al.* (1976)
Herpes 2	Mouse	i.d. (genital)	Allen *et al.* (1977)
Mareks disease	Chicken	s.c.	Eidson *et al.* (1974)
Equine abortion	Hamster	i.p.	Sidwell (1977)
Vaccinia	Rabbit	c.s. (eye)	Sidwell *et al.* (1972, 1973)
	Mouse	i.v. (tail)	De Clercq *et al.* (1976)
	Mouse	s.c. (tail)	Sidwell (1977); Sidwell *et al.* (1972)
	Rabbit	s.c. (back)	Sidwell (1977)
Fibroma	Rabbit	i.d.	G. Werner, cited by Sidwell *et al.* (1974)

[a]From Allen (12)
[b]c.s.: corneal scarification; i.d.: intradermal; i.n.: intranasal; i.p.: intraperitoneal; i.v.: intravenous; s.c.: subcutaneous

Lassa virus die between day 10 and 14 after inoculation. In contrast, all monkeys treated with ribavirin survive and peak viraemia titres are significantly lower than those of the surviving control monkeys. In a similar study (14) Rhesus monkeys infected with machupo viruses reached peak viraemia by days 12–14 and began to die. Treatment of individual monkeys with ribavirin was initiated at the time of onset of fever and was continued every 8h for 10 days, and death was prevented during the acute phase of the illness. The late neurological syndromes seen in about 20% of infected untreated control monkeys however, is not prevented by ribavirin treatment.

Toxicity studies

Over the past 15 years, extensive toxicity studies of ribavirin have been carried out in rats, mice, guinea pigs, rabbits, ferrets, dogs, baboons, rhesus and squirrel monkeys and cultured cells. The principal effect noted has been a fully reversible anaemia.

Acute toxicity studies

An oral LD_{50} (median lethal dose) of greater than 10 000 mg/kg was found in studies in mice and rhesus monkeys; a value of about half that was found in rats (11,15). Dogs appear to be the most sensitive species. The intraperitoneal LD_{50} in rodents ranges from about 25–60% of that seen orally. At lethal doses, death does

Table 5
RNA virus infections significantly inhibited in vivo *by ribavirin*[a]

Virus	Animal host	Route of virus inoculation [b]	References
Influenza A	Mouse	i.n.	Sidwell *et al.* (1972); Khare *et al.* (1973); Durr *et al.* (1975); Tisdale and Bauer (1975); Ensman *et al.* (1977); others cited by Sidwell *et al.* (1974)
Influenza A	Hamster	i.n.	Reins (1977)
Influenza	Ferret	i.n.	Schofield *et al.* (1972); Potter *et al.* (1976)
Influenza A	Monkey	i.n.	Stephen *et al.* (1977)
Parainfluenza 1	Mouse	i.n.	Sidwell, *et al.* (1972, 1975b); Larsson *et al.* (1978)
Parainfluenza 3	Hamster	i.n.	Sidwell *et al.* (1975b)
Murine hepatitis	Mouse	i.p.	G. Werner, cited by Sidwell (1977); Sidwell *et al.* (1974)
Rift Valley fever	Mouse	i.p.	Eddy *et al.* (1981)
Foot and mouth disease	Mouse	s.c.	G. Werner, cited by Sidwell *et al.* (1974)
West Nile	Mouse	i.p.	Odelola (1977)
Friend leukaemia	Mouse	i.p.	R. Gordes, cited by Sidwell *et al.* (1974); Sidwell *et al.* (1974); Sidwell *et al.* (1975a)
Moloney leukaemia	Mouse	i.p.	G. Werner, cited by Sidwell *et al.* (1974)
Rauscher	Mouse	i.p.	Shannon (1977); G. Werner, cited by Sidwell *et al.* (1974)
Gross (AKR) leukaemia	Mouse	Spontaneous	Bekesi *et al.* 1976
Lassa fever	Monkey	s.c.	Jahrling *et al.* (1980)
Machupo	Monkey	s.c.	Stephen *et al.* (1980)

[a]Adapted from Allen (12)
[b]i.n.: intranasal; s.c.: subcutaneous; i.p.: intraperitoneal

not usually occur until several days following dosing and is preceded by anorexia, lethargy and prostration. Pathological changes noted at necropsy are limited to signs of gastrointestinal haemorrhage and fluid accumulation (11,15).

Short term repeated dose studies

Toxicity studies of short term, repeated, daily dosing of 10–30 days duration have been conducted by the oral, inhalation and intramuscular (i.m.) routes of administration in ferrets, dogs, rats and monkeys. Similar to what was observed in the acute studies, dogs appear to be atypically sensitive; rats and monkeys are more tolerant (11,15–17). Full reversible anaemia is the major effect seen upon short term dosing. At very high oral doses in rats, reduced weight gains and feed

consumption have been reported. A small percentage of deaths were observed after repeated daily oral doses in the 200 mg/kg range. Minimal histopathological changes, primarily lymphoid depletion, have been observed at these elevated doses in rats, while gross changes have been limited to indications of small thymus, gastrointestinal haemorrhage and fluid accumulation (15). Indications of cardiac and testicular lesions seen in one study were not confirmed in subsequent studies. Primary histopathological changes in the gastrointestinal tract, bone marrow, spleen and thymus were observed in dog studies (11).

A pioneering inhalation toxicity study involving whole body exposure of suckling ferrets to ribavirin aerosols daily for up to 30 days was affected by lactation failure in the mothers, evidently from the stress of the high aerosol loading in the exposure chambers necessary to achieve the required aerosol concentrations (18). No ribavirin related histopathological changes were noted in the lung at doses about 5–27 times higher than therapeutic levels. Using quantitative morphometry, an approximately 20% increase in alveolar diameter was noted at 0–20 days post dosing, but not after 140 days. The significance of this observation is questionable since the increased diameters were within the mean values seen in different control groups. No treatment related changes were noted in studies of lung function and quantitative evaluation of the lavagable cell pool. A small increase in lung DNA/protein ratio was noted in the smaller kits but this effect disappeared after the recovery period.

Chronic/carcinogenicity studies

No statistically significant increase in tumours at any tissue site were reported in 2 life time carcinogenicity studies of ribavirin administered in the feed (19). Statistically non-significant increases in pituitary adenomas in males in the first study and mammary adenomas in females in the second study were, in each case, not found in the corresponding study. No consistent microscopic findings of drug related non-neoplastic changes were reported other than dose related increases in erythrocyte precursors in peripheral blood smears and reduction in myeloid elements in bone marrow smears. Increased regional hair loss was observed at high doses. Dose related decreases were reported in circulating red cell mass, food consumption and body weight gain (15,19).

Genetic toxicity studies

No genotoxicity was seen in microbial mutagenicity studies in Salmonella and dominant lethal studies in mice and rats (15,19,20). No cytogenetic effects of ribavirin were seen in studies of rat bone marrow cells *in vivo* and rat fibroblast cells *in vitro* (15,19). An increase in mutations at the thymidine kinase locus was observed in mouse lymphoma cells cultured *in vitro* in the presence of ribavirin. The addition of liver activating enzymes markedly reduced the response to ribavirin in the mouse lymphoma system (Unpublished report, 1982, No. 20989). However, the result is difficult to interpret since artifacts could result because ribavirin inhibits thymidine kinase activity and normal DNA bases and nucleosides also cause apparent increases in mutations in cultured cells (21,22).

An increase in transformation rate of BALB 3T3 cells cultured in the presence of

ribavirin was seen at only one test concentration (15 μg/ml) but not at twice or one-half that value (Unpublished report, 1982, LBI No. 20992). Acyclovir also induced transformations in this system as well as giving an apparent mutagenic response at the TK locus in mouse lymphoma cells, yet it was found non-carcinogenic in animal carcinogenicity studies (24,25). A normal constituent of DNA, 2-deoxyguanosine, is reportedly mutagenic in both mouse lymphoma and BALB 3T3 cells (24).

Reproductive studies

Fertility studies in rats have not indicated any adverse effects except for a possible decrease in fertility following i.p. injection of nearly lethal to lethal doses in males (20).

Dose related increases in external anomalies such as gastroschisis, exencephaly and eye defects have been observed in teratology studies carried out in rats, mice and hamsters (15,19,26). Abnormalities in skeletal development and foetotoxicity have also been reported in rodents. Foetotoxicity has been observed in rabbits. A teratology study in baboons at oral doses of 60 and 120 mg/kg/day given during organogenesis indicated no teratogenic or foetotoxic effects (15,19).

Special studies

Special studies have been carried out to investigate the haematological effects of ribavirin (11,17,27,28). Large amounts of ribavirin are sequestered in RBC's (11,23). Storage may be due in part to the conversion of ribavirin to the phosphorylated nucleotide inside the RBC. Studies *in vitro* indicated that monkey RBC's take up the largest amount of ribavirin, human RBC's take up an intermediate amount and rat RBC's the least (28). Studies of RBC lysis in hypotonic salt solutions and deformability measured by passage through a 3μ filter indicated that ribavirin uptake did not alter RC fragility or deformability.

Studies with red cells labelled with ^{3}H-disopropyl fluorophosphate and injected back into Rhesus monkeys receiving 0, 15 or 60 mg/kg/day of ribavirin daily by i.m. injection indicated a dose related removal of RBC's from the circulation in treated animals (16). The half life of the labelled cells in ribavirin treated animals returned to control values 2 weeks after discontinuation of ribavirin treatment. Extravascular haemolysis was concluded to be the cause of the red cell removal because ribavirin treatment does not increase serum bilirubin or decrease haptoglobin. Furthermore, *in vitro* treatment with 4 mM ribavirin does not alter the survival of labelled RBC's injected back into the monkeys nor does it increase susceptibility to lysis from osmotic insult. Doses of 60 mg/kg resulted in a decrease in specific activity of labelled cells following, but not preceding, the end of dosing indicating that ribavirin at this level inhibits release of reticulocytes from the bone marrow. This is substantiated by the marked reticulocytosis that develops post dosing. Thus ribavirin did not produce anaemia by damage to stem cells, but rather by extravascular haemolysis and a delay in maturation.

Further studies were conducted on monkeys given a dose regimen of 0, 30 or 100 mg/kg/day of ribavirin by i.m. injection for 10 days (16,17). In addition to a reversible decrease in circulating red cell mass, reversible dose related throm-

bocytosis was observed along with increased platelet size at the high dose. No changes in platelet function were observed. Bone marrow aspirates revealed a reversible increase in myeloid to erythroid ratio. Early erythroid precursors were either unchanged or increased as a result of treatment, whereas the late erythroid precursors were decreased. Myeloid cell numbers were not affected by treatment. Plasma cells and histocytes were increased and increased phagocytosis of RBC's by bone marrow histocytes was observed in treated animals. Vacuolization of marrow cells was noted, especially in cells of the erythroid series. These studies also suggest that ribavirin produces a fully reversible anaemia due to extravascular haemolysis and, at high doses, by a delay in red cell maturation.

Metabolism of ribavirin

Ribavirin until recently has been exceedingly difficult to quantitate physiologically. Its UV absorption spectrum has not led to sensitive measurements (Max at 207 nm; extinction coefficient equalled to 1.17×10^{-3} l/mol/cm) of metabolites and unchanged compound. Most pharmacokinetic studies have depended upon the use of either ^{14}C or ^{3}H labelled material and have been reported in terms of radioactivity in tissue and fluids. However, recently a radioimmunoassay has been developed for the detection of ribavirin in biological fluids (29). Early studies in rats (30) suggested that ribavirin is both deaminated and deribosylated, and thus both the ribosylated and nonribosylated triazolecarboxamide were seen in urine. However, when 5-^{14}C- ribavirin was administered to rats very little (approximately 3%) of the ^{14}C was expired as $^{14}CO_2$ (31). Thus the triazole ring may be considered metabolically stable. In humans, 53% of orally administered ribavirin was eliminated in 72h, and like Rhesus monkeys humans were shown to concentrate ribavirin in red blood cells. At 72h about 3% of the total dose administered is present in human red cells (32). The later phase of the plasma disappearance curve is 48h or more. Unlike rats, where the deaminated form of ribavirin shows up in the urine, our studies employing a high performance liquid chromatography technique have shown only 1,2,4-triazolecarboxamide and unchanged ribavirin in the urine in nine subjects treated with ^{14}C ribavirin. Figs. 2 and 3 show the results obtained from two of the subjects, although all nine were uniform with respect to products found. A more recent study during this year employed the radioimmunoassay discussed previously. Connor *et al.* in 1985 administered ribavirin orally to 17 asymptomatic homosexual men with lymphadenopathy syndrome and revealed mean plasma half lives of approximately 2h for the alpha phase and 36h for the beta phase. The volume of distribution was approximately 650 litres. The mean plasma ribavirin concentrations which occurred 1.5h after dosing were 5.19 and 12.6 μM, respectively, following doses of 600, 1200 and 2400 mg p.o. It is clear from this study that ribavirin is incompletely absorbed from the intestinal tract.

Mechanisms of action

Ribavirin is phosphorylated in red blood cells and, as expected, is rapidly converted to its 5′ triphosphate. Ribavirin in red blood cells principally exists as the 5′ triphosphate and the ratio of tri: di: monophosphate is about the same as that of adenosine nucleotides (25 to 5 to 1) (23). Human red blood cells are known to be