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SYNERGISTIC CYTOTOXIC EFFECT OF TIAZOFURIN AND RIBAVIRIN IN HEPATOMA CELLS

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Tiazofurin, an anti-cancer drug, which induces remissions in human leukemia, and ribavirin, an anti-viral agent, bind at separate sites (NADH and IMP-XMP sites, respectively) on the target enzyme, IMP dehydrogenase. Now we show that the binding to IMP dehydrogenase of these drugs at two separate sites is translated into synergistic inhibition of de novo guanylate biosynthesis and synergistic toxicity in rat hepatoma 3924A cells. These results may be utilized in the chemotherapy of neoplastic diseases and in the treatment of hepatitis virus infection and hepatocellular carcinoma.

IMP dehydrogenase (EC 1.1.1.205), the rate-limiting enzyme of de novo GTP biosynthesis, is a sensitive target in anti-cancer and anti-viral chemotherapy (1-5). Ribavirin is a potent anti-viral agent with a broad spectrum including hepatitis B virus (6) and tiazofurin has wide murine anti-tumor activity with low anti-viral action (Fig. 1). The recent demonstration of the striking action of tiazofurin in human leukemia has increased interest in drugs directed against IMP dehydrogenase (7-9). Kinetic studies in IMP dehydrogenase purified to homogeneity from rat hepatoma showed that tiazofurin through its active metabolite, TAD, inhibits the enzyme at the NADH site, whereas ribavirin through its metabolite, RMP, blocks at the IMP-XMP site (10). Preincubation with RMP increases the inhibition in a time-dependent manner and the presence of TAD promotes further inhibition (10). The present novel results show that ribavirin and

The abbreviations used are: RMP, ribavirin 5'-monophosphate; TAD, thiazole-4-carboxamide adenine dinucleotide.

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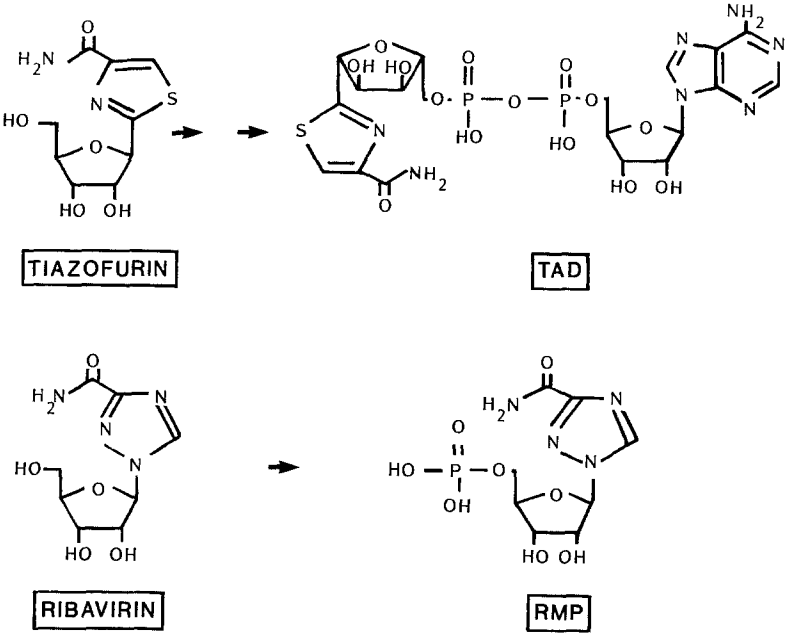

Fig. 1 Structures of tiazofurin, ribavirin and their active forms. In sensitive cells, tiazofurin is phosphorylated to tiazofurin 5'-monophosphate which is anabolized by NAD pyrophosphorylase to TAD; ribavirin is phosphorylated to RMP.

tiazofurin synergistically inhibit the conversion of IMP to guanylates and provide synergistic cytotoxicity in hepatoma cells. This should be important in achieving better anti-cancer chemotherapy and may combine the action against hepatitis virus with an effect on hepatocellular carcinoma in human.

MATERIALS AND METHODS

Materials. [^{14}C]Formate was obtained from Amersham. Bactopeptone-free McCoy's 5A medium and tissue culture supplies were purchased from Grand Island Biological Co. Eagle's basal medium containing Earle's salt, L-glutamine and HEPES was from Sigma.

Cell Culture. Rat hepatoma 3924A cells in monolayer culture were maintained in log phase in McCoy's 5A medium supplemented with 10% fetal calf serum, penicillin, 100 units/ml and streptomycin, 100 mg/ml (11). Clonogenic assays were performed in McCoy's 5A medium with dialyzed fetal calf serum without bactopeptone to be free from nucleosides and nucleobases. After 7 days of exposure to drugs colonies of more than 50 cells were counted (12).

Assays of de Novo Purine and Guanylate Synthesis in Hepatoma Cells. The initial rates of de novo purine synthesis were determined as outlined elsewhere (13). Serine-free medium was used in the assays to avoid dilution of purine labeling with one-carbon units from serine. The cells were pulsed with 4 mM [^{14}C]formate, purine compounds were hydrolyzed to purine bases and the labeled purines were separated from other products by Dowex 50-H^+ column and two-dimensional paper chromatography (13).

RESULTS AND DISCUSSION

Determination of Rates of de Novo Guanylate Synthesis. [^{14}C]Formate initially labels IMP among purine compounds. IMP is metabolized by 4 enzymes: adenylosuccinate synthase, IMP dehydrogenase, 5'-nucleotidase and hypoxanthine phosphoribosyltransferase (reverse reaction). The latter two enzymes degrade IMP to inosine and hypoxanthine which are not directly utilized for the production of adenylates and guanylates in mammalian cells. Only at the nucleotide level is IMP interconvertible to adenine and guanine nucleotides. The labelings of adenine and guanine as well as total purine labeling were linear with incubation time up to 60 min (Fig. 2A). Since the labeled purines were fully recovered from all purine compounds including nucleic acids, the incorporation rates of [^{14}C]formate into guanine should reflect the flux of de novo guanylate synthesis via IMP dehydrogenase activity in hepatoma cells.

Effects of Drugs on de Novo Purine and Guanylate Synthesis in Hepatoma 3924A Cells. Treatment of hepatoma cells with ribavirin at a final

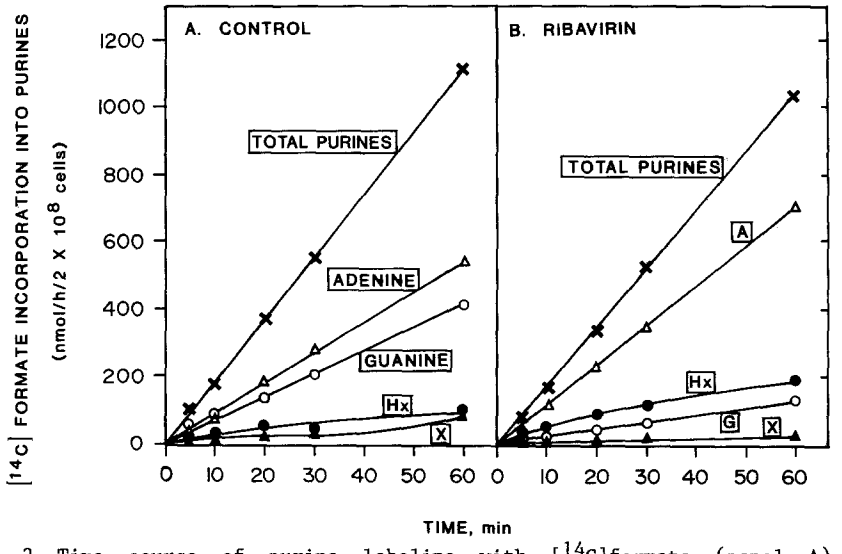

Fig. 2 Time course of purine labeling with [^{14}C]formate (panel A) and effect of ribavirin on the label distribution (panel B). Hepatoma 3924A cells in log phase were treated with ribavirin at 100 uM for 2 h. The non-treated control cells (panel A) and the drug-treated cells (panel B) were then pulsed with [^{14}C]formate. The labeled purines were hydrolyzed, separated and counted as described in "MATERIALS AND METHODS." A, adenine; Hx, hypoxanthine; G, guanine; x, xanthine.

concentration of 100 uM for 2 h affected the label distribution of purines without significantly changing the rates of purine de novo synthesis (Fig. 2B). The decrease of labels in guanine and xanthine accompanied by the increase of those in adenine and hypoxanthine supports the concept that IMP dehydrogenase is the primary target of ribavirin. Treatment with tiazofurin (100 uM) for 2 h exerted similar effects. Tiazofurin and ribavirin additively inhibited de novo guanylate synthesis at 2 h after treatment (not shown); when the drugs were used in combination in 48 h treatment, synergistic inhibitory action was observed (Table 1). When cells were treated for 48 h with 10 uM ribavirin or 8 uM tiazofurin, the rates of de novo guanylate synthesis decreased to 30 and 52%, respectively, of that in controls. A combination of the two drugs resulted in 6% of control value which indicated synergism, since a summation would have yielded 20% (Table 1).

In our previous study RMP plus TAD additively inhibited IMP dehydrogenase purified to homogeneity from hepatoma 3924A (10). However, the enzyme was

Table 1: Effect of ribavirin and tiazofurin on de novo purine and guanylate syntheses in hepatoma 3924A cells

	Purine de novo synthesis: nmol/h/2 x 10^8 cells (% of control)			
Drugs, uM	Total purine		Guanylates	
	Determined	Predicted	Determined	Predicted
None, control	246 ± 7 (100)		85 ± 4 (100)	
Ribavirin, 10	231 ± 19 (94)		33 ± 5 (39)*	
Tiazofurin, 8	211 ± 4 (86)*		44 ± 2 (52)*	
Ribavirin, 10 + Tiazofurin, 8	190 ± 6 (77)*	(81)	5 ± 1 (6)*+	(20)

Means ± S.E. of triplicate assays are given. Hepatoma 3924A cells in log phase were seeded at a population of 3 X 10^5 cells for control or 5 X 10^5 cells for drug treatment per 25 cm^2 flask. The cells were treated with drugs for 48 h. In the assays of purine de novo synthesis the cell number in each flask was 3.6 ± 0.2 x 10^6 cells.

* Significantly different from control ($p < 0.05$).

+ Significant synergism, determined value < 70% of the predicted value.

synergistically inhibited by preincubation with RMP and TAD at 37°C prior to the assay, since TAD promoted a time-dependent inhibition of IMP dehydrogenase by RMP (10). In intact cells the drug actions might be affected by several other factors including drug transport, metabolism and intracellular compartmentation. Tiazofurin inhibits transport of nucleosides into cells (14); it is likely that it would compete with ribavirin, a nucleoside, for drug uptake. Thus, it requires time to convert the drugs to the active forms, accumulate to the effective intracellular concentrations and exert synergistic inhibition of IMP dehydrogenase in the cells.

Synergistic Cytotoxicity of Ribavirin and Tiazofurin in Hepatoma Cells. The effects of IMP dehydrogenase inhibitors on the survival of hepatoma 3924A cells were investigated in clonogenic assays. Treatment of cells for 7 days with tiazofurin, ribavirin and selenazofurin, a selenazole analogue of tiazofurin, yielded LD_{50} of 6.5, 12 and 1.5 uM, respectively. Treatment with 6 uM tiazofurin or 10 uM ribavirin resulted in a survival of 56 and 70%, whereas a combination of the two drugs yielded 18% survival, indicating synergistic action (Table 2). Selenazofurin plus ribavirin also exerted synergistic killing (Table 2). These observations support a combination chemotherapy targeted against different ligand sites of IMP dehydrogenase, also because the concentrations of tiazofurin and ribavirin are well within the plasma range readily achievable in clinical treatment (15,16). Since the mechanism of action of selenazofurin through selenazole-4-carboxamide adenine dinucleotide, an NAD analogue, (17) has the same inhibitory mechanism as TAD on IMP dehydrogenase (18) tiazofurin plus selenazofurin produced only additive inhibition.

Tiazofurin is useful in the treatment of human leukemia because of its selective action on leukemic blasts (7,8), as an inducer of differentiation (7,8,19,20), the availability of a rapid predictive test for sensitivity of leukemic cells to the drug (21) and monitoring the drug action by measuring IMP dehydrogenase activity (9). The primary target of tiazofurin is IMP dehydrogenase, the rate-limiting enzyme for biosynthesis of guanine

Table 2: Effects of IMP dehydrogenase inhibitors on survival of hepatoma 3924A cells

Drugs, uM	% of Control survival	
	Observed	Predicted
Control	100	
Tiazofurin, 6	56 ± 6	
Selenazofurin, 1	63 ± 3	
Ribavirin, 10	70 ± 4	
Tiazofurin, 6 + Selenazofurin, 1	38 ± 4	35
Tiazofurin, 6 + Ribavirin, 10	18 ± 0*	39
Selenazofurin, 1 + Ribavirin, 10	15 ± 1*	44

Means ± S.E. of 9 or more assays are given. Cells were treated for 7 days and survival was measured by colony counts.

* Significant synergism, observed value < 70% of the predicted value.

nucleotides, which are involved in many important biological functions including synthesis of RNA, DNA and protein, signal transduction and polymerization of tubulin (9,22). The cytotoxic action of tiazofurin is stringently linked with the decrease of GTP concentrations in hepatoma cells (1-4). The present study suggests that ribavirin might be useful in combination chemotherapy with tiazofurin to strengthen the blocking of de novo guanylate synthesis and in the treatment of hepatitis virus infection coupled with hepatocellular carcinoma.

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