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ENZYME PATTERN-DIRECTED CHEMOTHERAPY

EFFECTS OF ANTIPYRIMIDINE COMBINATIONS ON THE RIBONUCLEOTIDE CONTENT OF HEPATOMAS*

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Abstract—Using combinations of three antipyrimidine drugs, chosen on the basis of expected antihepatoma selectivity, we examined the effects on pyrimidine ribonucleotide pools in rat hepatomas (Morris 8999 and 3924A) and host livers. D-Galactosamine gave a marked decrease in UTP in hepatomas, 3-deazauridine caused decreases in CTP concentration, and these two agents together gave very pronounced (up to 86 per cent) decreases in hepatoma CTP content. Pyrazofurin caused decreases in UTP in both hepatoma and host liver, but in combination with D-galactosamine, pyrazofurin decreased hepatoma CTP by up to 79 per cent without decreasing the host liver CTP. The combination of pyrazofurin and D-galactosamine also gave a significantly greater depression of UTP levels in hepatoma 8999 than in host liver. Uridine and orotic acid both restored the D-galactosamine-induced depletion of UTP in liver and hepatoma 8999.

In recent experimental chemotherapy studies with rat hepatomas, an approach was tested in which the pattern of enzyme activities characteristic of hepatoma tissue was exploited to enhance selectively the cytotoxicity of combinations of antipyrimidine drugs in hepatoma cells [1, 2]. For example, the amino-sugar D-galactosamine ($GalNH_2$)† is selectively toxic to hepatic tissue, and this selectivity has been attributed to the fact that galactokinase (EC 2.7.1.6) and UDPG:galactose-1-phosphate uridyltransferase (EC 2.7.7.12) have predominantly hepatic activities [3]. By the combined action of these two enzymes, $GalNH_2$ produces severe depletion of the hepatic UTP pool *in vivo* [4]. In some hepatomas, the galactokinase and UDPG:gal-1-P uridyltransferase are retained to a significant extent, and thus, $GalNH_2$ is toxic at low concentration in these neoplastically transformed hepatic cells, but not in nonhepatic proliferating cells [1].

3-Deazauridine (3-DAU) in the form of its 5′-triphosphate (3-DAUTP) is a potent inhibitor of CTP synthetase (EC 6.3.4.2) [5, 6]. Previous work in this laboratory showed that $GalNH_2$ and 3-DAU gave synergistic growth inhibition of rat hepatoma cells *in vitro*, but in nonhepatic lines (fibroblasts and leukemia cells) higher $GalNH_2$ concentrations were required to obtain inhibition, and combined effects were only additive, not synergistic. The selectivity of this combination toward hepatoma cells was attributed to the inhibitory effect of 3-DAUTP being enhanced in these cells by the $GalNH_2$-induced depletion of UTP.

The effect of $GalNH_2$ on UTP pools is reduced to some extent in hepatoma cells, relative to normal liver. This may be attributable to the fact that *de novo* pyrimidine biosynthesis is more active in the malignantly transformed cells [7, 8] which enables the hepatoma to replace the lost UTP rapidly.

It thus appeared that the efficacy of $GalNH_2$ might be enhanced by using it in combination with an inhibitor of the *de novo* pyrimidine biosynthetic pathway. Pyrazofurin (PF) is a C-nucleoside antibiotic isolated from the fermentation product of *Streptomyces candidus* [9]. PF has been shown to inhibit the growth of some rodent tumors [10]. It is converted to its active form, the 5′-monophosphate (PFMP), by the action of adenosine kinase [11]. PFMP is an inhibitor of orotidylate decarboxylase [9]. Hepatomas have higher activity of adenosine kinase than any of the normal proliferating cells of the body [12] which suggests that PF might be an effective agent for chemotherapy of hepatomas, particularly in combination with $GalNH_2$ or 3-DAU. The relationships of the metabolic sites inhibited by the three agents are summarized in Fig. 1. The present paper describes the effects of these agents, alone or in combination, on the nucleotide contents of hepatomas and host liver.

Orotic acid is able to enter cells of the liver freely, but the permeability to orotic acid is greatly impaired in hepatomas [13, 14]. This suggested the possibility of a selective "rescue" of liver from the effects of high doses of $GalNH_2$ by using orotate, which should be less effective at reversing the $GalNH_2$ effect in hepatomas, by virtue of its limited uptake into the malignant cells. Experiments designed to test this concept are described.

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† Abbreviations used: $GalNH_2$, D-galactosamine; 3-DAU, 3-deazauridine; 3-DAUTP, 3-deazauridine 5′-triphosphate; PF, pyrazofurin; PFMP, pyrazofurin 5′-monophosphate; UDPGA, UDP-glucuronic acid; and UDPAG, UDP-*N*-acetylglucosamine.

MATERIALS AND METHODS

Materials. 3-Deazauridine was purchased from the ICN Life Sciences Group, Cleveland, OH, and D-galactosamine from the Sigma Chemical Co., St. Louis, MO. Pyrazofurin was generously donated by Eli Lilly & Co., Indianapolis, IN. Tissue culture supplies were

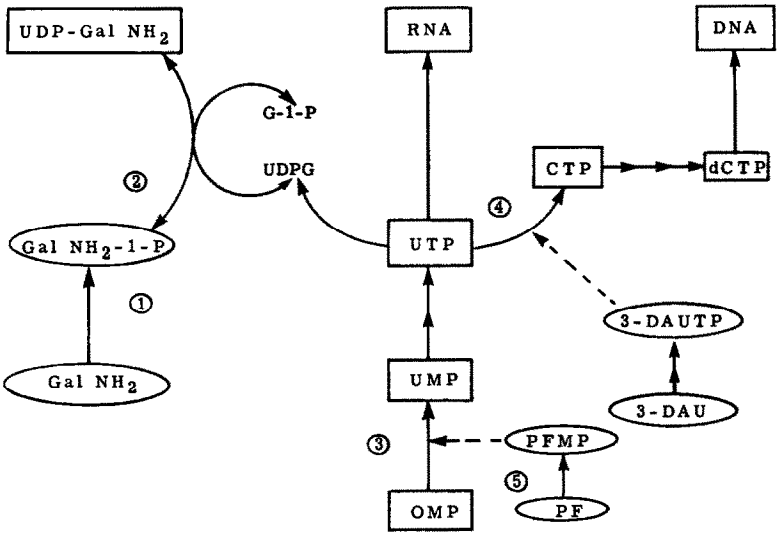

Fig. 1. Simplified scheme of pyrimidine biosynthesis, showing sites of action of D-galactosamine, 3-deazauridine and pyrazofurin. The numbered enzymes are: (1) galactokinase; (2) UDPG:gal-1-P uridyltransferase; (3) orotidylate decarboxylase; (4) CTP synthetase; and (5) adenosine kinase.

obtained from Grand Island Biological Co., Grand Island, NY, or from Flow Laboratories, Rockville, MD. Reagent chemicals were purchased from the Fisher Scientific Co., Fair Lawn, NJ, or from the J. T. Baker Chemical Co., Phillipsburg, NJ.

Cell culture. The origin and properties of the 8999R cultured hepatoma line have been described [1, 2]. The cells were grown as monolayer cultures in Falcon tissue culture flasks (Falcon Plastics, Oxnard, CA) in McCoy's medium 5A, supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were subcultured at weekly intervals, and medium was changed midway between successive subcultures. The log phase doubling time under these conditions was 40 hr.

Animal tumors. The tumors were Morris solid hepatomas 8999 and 3924A. Two groups of hepatoma 8999 were used; generation 23, which had an interval between transplants of 6.3 months, and generation 32, in which the transplant interval had decreased to 3.5 months. These 8999 hepatomas were carried as bilateral subcutaneous transplants in male inbred Buffalo rats (6-weeks-old, 200 g). Hepatoma 3924A is a more rapidly growing, poorly differentiated hepatoma, with a transplant interval of 4 weeks; the 3924A hepatoma was carried as a bilateral subcutaneous transplant in male inbred ACI/N rats (4-weeks-old, 100 g). Animals were kept in separate cages with water and Purina laboratory chow available *ad lib.* Tumors were used when they reached a diameter of about 1.5 cm.

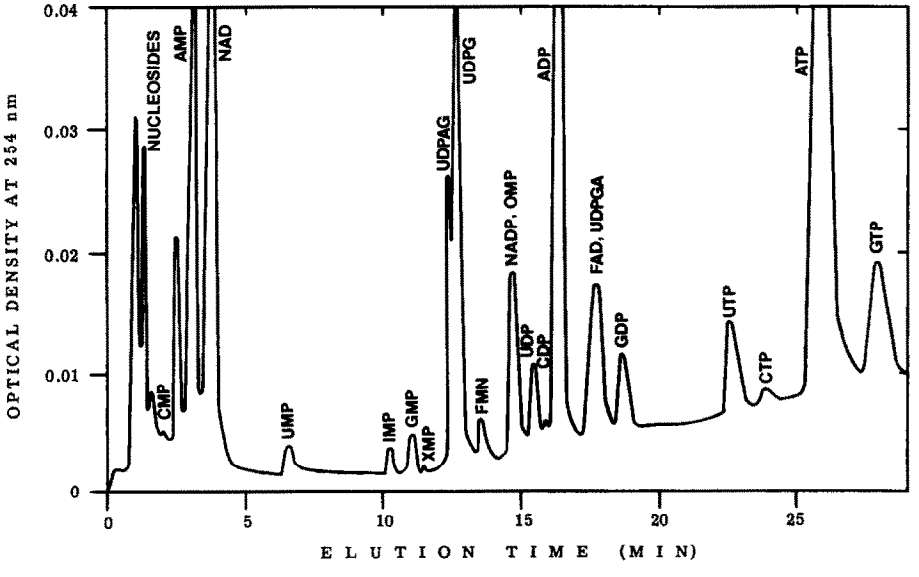

Fig. 2. Separation of ribonucleotides by anion exchange chromatography. The sample shown was an extract of control rat liver. Extraction and analysis were as described in Materials and Methods.

Table 1. Effects of antipyrimidines on ribonucleotide pools in cultured hepatoma cells (8999R)*

Nucleotides	Control (no treatment)	3-Deazauridine (0.3 μM)	D-Galactosamine (0.3 mM)	3-Deazauridine (0.3 μM) plus D-galactosamine (0.3 mM)
ATP	5530	4760	6770	6090
ADP	980	1210	873	805
GTP	1870	1940	1770	1560
GDP	353	462	336	315
UTP	1690	1280	348†	397†
UDP	224	238	125†	139†
CTP	563	282†	385	77‡
Growth (% control)	100	48	53	16

* Values are nmoles/10^9 cells (means of triplicate cultures). Treatment was for 16 hr.
† Significantly different from untreated ($P < 0.05$).
‡ Significantly different from 3-deazauridine- or D-galactosamine-treated ($P < 0.05$).

Tissue extraction. Rats were anesthetized with ether; livers and hepatomas were exposed, and freeze-clamped rapidly in liquid nitrogen cooled tongs [15, 16]. The frozen tissue was ground in a liquid nitrogen cooled mortar, and 0.5 g of the powder was extracted in 4 ml of 0.5 M perchloric acid. After centrifugation to remove precipitated protein, the extract was neutralized with 4 N potassium hydroxide. Aliquots of the neutralized extract (usually 50 μl) were used for nucleotide analysis.

Nucleotide analysis. Nucleotides were separated by anion exchange, using a Varian model 8520 high pressure liquid chromatograph. A 4.6 mm × 25 cm column of Partisil SAX (Whatman, Clifton, NJ) was eluted with a starting buffer of ammonium phosphate, pH 2.8, 0.005 M with respect to phosphate. After a gradient delay of 4 min, 0.5 M ammonium phosphate, pH 5.0, was mixed with starting buffer at a concentration increasing by 5 per cent/min. Total flow rate was 120 ml/hr, column temperature was 23°, and pressure was 10.6 MPa (about 1500 psi). Peaks were detected by u.v. absorbance at 254 nm, and were integrated by the cut-and-weigh procedure, with calibration factors measured by known amounts of pure compounds. A sample chromatogram obtained with a control rat liver extract is shown in Fig. 2.

Statistical evaluation of results. The experimental results were subjected to statistical evaluation by means of the 2-tail Student's *t*-test. Differences between means giving a probability of less than 5 per cent were considered to be significant.

RESULTS

Effects of 3-DAU and $GalNH_2$, alone and in combination, on the ribonucleotide contents of cultured 8999R hepatoma cells are shown in Table 1. The drug concentrations chosen were sublethal, and close to the ID_{50} values, i.e. concentrations that gave 50 per cent growth inhibition over one generation time, relative to untreated control cultures. Drug treatment was for 16 hr. These experimental conditions thus made it possible to examine steady state nucleotide concentrations in cells which, though inhibited, were healthy and actively proliferating throughout the period of the study. This approach eliminates the possibility that perturbations seen in the treated cells were secondary consequences of cell lysis or other lethal damage. 3-DAU at 0.3 μM caused 50 per cent reduction in the steady state CTP pool. Only minor changes were seen in purine ribonucleotide pools. $GalNG_2$ at 0.3 mM caused a large (79 per cent) depression in UTP, and a 32 per cent depression in CTP. No significant changes in purine ribonucleotide pools. $GalNH_2$ at 0.3 mM treatment. It is interesting that these $GalNH_2$-treated cells were able to multiply at 50 per cent of the control rate, though UTP concentration was only 21 per cent of normal, whereas a 50 per cent reduction in CTP (caused by 3-DAU) gave a 50 per cent reduction in growth rate. The final column of Table 1 shows the effects of a combination of 3-DAU (0.3 μM) plus $GalNH_2$ (0.3 mM). Since each individual agent gave 50 per cent growth inhibition at these concentrations, the

Table 2. Effects of D-galactosamine and 3-deazauridine on pyrimidine ribonucleotide contents of hepatoma 8999 *in vivo**

Nucleotides	Control (no treatment)	D-Galactosamine (360 mg/kg)	3-Deazauridine (200 mg/kg)	D-Galactosamine (360 mg/kg) plus 3-Deazauridine (200 mg/kg)
UDP	92	69	123	78
UTP	301	134†	219	161†
CTP	71	37	26†	12†

* Drugs were administered i.p. and hepatomas were freeze-clamped after 2.5 hr. Values are means of three rats in each group, expressed as nmoles/g wet weight.
† Significantly different from untreated ($P < 0.05$).

Table 3. Effects of uridine and orotate in combination with $GalNH_2$ on UDP, UTP and CTP concentration in normal rat liver*

Treatment	UDP (% of control)	UTP (% of control)	CTP (% of control)
Control (no treatment)	100	100	100
Galactosamine (330 mg/kg)	89	15†	112
Galactosamine (330 mg/kg) and uridine (730 mg/kg)	240†	166†	121
Galactosamine (330 mg/kg) and orotate (230 mg/kg)	342†	495†	236†

* Means of three or more rats are provided. Galactosamine was given i.p. 19 and 2 hr before killing. Galactosamine and uridine were injected i.p. 21, 6 and 2 hr before killing. Galactosamine and orotate were administered i.p. 21 and 6 hr before killing.

† Significantly different from untreated ($P < 0.05$).

growth rate predicted in the presence of the combination, on a basis of therapeutic summation, was 25 per cent of control; in fact the measured growth rate was 16 per cent of control, the two agents showing therapeutic synergism, in agreement with previous observations [1]. This 84 per cent reduction in growth rate caused by the 3-DAU + $GalNH_2$ combination was accompanied by an 86 per cent decrease in cellular CTP content. The depression of UTP by the combination was approximately equal to that given by $GalNH_2$ alone, but it is clear that simultaneous inhibition of CTP synthetase by 3-DAUTP and depletion of the competing substrate (UTP) by $GalNH_2$ cause a very marked decrease in cellular CTP, and this is accompanied by pronounced growth inhibition. Again, no significant effect was observed on the purine ribonucleotide pools.

Table 2 shows results of an analogous experiment conducted *in vivo*. The solid neoplasm in which these measurements were conducted (hepatoma 8999) is the tumor from which the 8999R cell line (used for the *in vitro* studies described above) was derived. Buffalo rats were injected intraperitoneally with 3-DAU and $GalNH_2$, alone and in combination. After 2.5 hr, hepatoma samples were taken by the freeze-clamp technique, and analyzed for UDP, UTP and CTP by high pressure liquid chromatography. Galactosamine alone at the dose used gave a 55 per cent depression of UTP, a 25 per cent decrease in UDP, and a 48 per cent decrease in CTP. 3-DAU gave a 27 per cent decrease in UTP, a 34 per cent increase in UDP, and a 63 per cent decrease in CTP. The two agents used in combination gave a 47 per cent decrease in UTP, a 15 per cent decrease in UDP, and an 83 per cent decrease in CTP. Thus, the primary effect of $GalNH_2$ was on UTP, the primary effect of 3-DAU was on CTP, and the two agents used in combination gave a depression in cellular UTP content which was similar to that of $GalNH_2$ alone, but a very much more pronounced depression of CTP than that given by 3-DAU alone. These findings parallel the results obtained *in vitro*.

Table 3 summarizes some data showing the effects of $GalNH_2$ on ribonucleotides in the liver of normal rats. As found with hepatoma 8999 *in vivo*, $GalNH_2$ caused a pronounced decrease in UTP content, but in the case of the liver no change was seen in the CTP level after $GalNH_2$ treatment (note the difference in dosage in the two experiments). Simultaneous administration of uridine prevented the $GalNH_2$-induced depletion of UTP, and indeed resulted in somewhat higher than normal UTP levels. Simultaneous administration of sodium orotate with the $GalNH_2$ not only prevented any deple-

Table 4. Effects of uridine and orotate in combination with galactosamine on UTP concentrations in hepatoma 8999 and host liver*

Treatment	UTP	
	Hepatoma 8999 (% of control)	Host liver (% of control)
Control (no treatment)	100	100
Galactosamine (330 mg/kg)	56	18†
Galactosamine (330 mg/kg) and uridine (730 mg/kg)	96‡	80‡
Galactosamine (330 mg/kg) and orotate (267 mg/kg)	128‡	39‡

* Means of three or more rats are given. Galactosamine was injected i.p. 18 and 1.5 hr before killing. Galactosamine and uridine were given i.p. 22.5 and 5 hr before killing.

An extra dose of uridine was administered 3 hr before killing. Galactosamine and orotate were given i.p. 18 and 1.5 hr before killing.

† Significantly different from untreated ($P < 0.05$).

‡ Significantly different from galactosamine-treated ($P < 0.05$).

Table 5. Effects of orotate on UTP concentrations in hepatoma 3924A and host liver*

Treatment	UTP	
	Hepatoma 3924A (% of control)	Host liver (% of control)
Control (no treatment)	100	100
Galactosamine (330 mg/kg)	75	22†
Galactosamine (330 mg/kg) and orotate (267 mg/kg)	81	54†

* Means of three or more rats are given. Drugs were administered i.p. 16 and 2 hr before killing.
† Significantly different from untreated ($P < 0.05$).

tion of UTP, but resulted in very marked overshoot of UTP (to 495 per cent of the normal value), and this effect was also accompanied by a significant elevation of hepatic CTP content.

Table 4 shows the effects of uridine and orotate in blocking the $GalNH_2$-induced UTP depletion in hepatoma 8999. As observed with the liver of normal rats (Table 3), $GalNH_2$ alone decreased the hepatoma UTP content, uridine when administered simultaneously with $GalNH_2$ maintained the UTP level near normal, and orotate caused an overshoot in UTP content. In this experiment with tumor-bearing rats, orotate did not prevent the depletion of host liver UTP by $GalNH_2$; this result is in contrast with the result obtained with liver of normal rats (Table 3), although there were slight differences in the length of treatment. The liver of tumor-bearing rats had been shown previously to exhibit biochemical properties different from those of normal liver [17].

Results with the rapidly growing, poorly differentiated hepatoma 3924A are shown in Table 5. In this highly malignant tumor $GalNH_2$ was comparatively ineffective. This is in agreement with results obtained with hepatoma 3924A in culture [1], where $GalNH_2$ caused relatively little growth inhibition, in comparison with the more slowly proliferating, better differentiated hepatoma 8999. In rats carrying the hepatoma 3924A, orotate was able to partially prevent the $GalNH_2$-induced decrease in UTP of the host liver.

The above experiments indicated that $GalNH_2$ usually caused a smaller decrease in the UTP content of the tumors than in the liver, and this might be attributed to the greater activity of the pyrimidine *de novo* biosynthetic pathway in the hepatomas, compared to liver, giving the malignant tissue the ability to replace the depleted UTP more rapidly than the liver. This assumes that the measured pyrimidine biosynthetic activities in liver reflect the activities of the predominant cell type (hepatocytes). To circumvent this problem, we tested the effect of combinations of $GalNH_2$ with PF. Since PF is activated by adenosine kinase [11], an enzyme which is very active in most well-differentiated hepatomas [12], it seemed to be a relatively specific agent to use for this purpose. Results obtained using the $GalNH_2$ plus PF combination against the hepatoma 8999 *in vivo* are shown in Table 6. PF alone gave comparatively small decreases in UTP and CTP in both tumor and host liver. However, PF in combination with $GalNH_2$ gave marked decreases in tumor UTP and CTP content, while giving a much less extensive depression of host liver UTP and no significant change in host liver CTP. Thus, the $GalNH_2$ plus PF combination appears to possess a considerable measure of antihepatoma selectivity. Similar experiments were also done with the more malignant hepatoma 3924A, and results are shown in Table 7. In this tumor the combination was, as expected, less effective than in the more highly differentiated hepatoma 8999, but again PF plus $GalNH_2$ gave a significant depression in tumor CTP content, without decreasing the CTP level of the host liver.

DISCUSSION

One of the constraining factors in the design of successful anticancer therapy with antimetabolite drugs continues to be their limited selectivity. Many of the agents at present in use are toxic to all proliferating cells, both malignant and non-malignant. However, while neoplastically transformed cells do not show any

Table 6. Effects of pyrazofurin and galactosamine on UTP and CTP concentration in hepatoma 8999 and host liver*

Treatment	UTP	CTP	UTP	CTP
	Hepatoma 8999		Host liver	
	(% of control)	(% of control)	(% of control)	(% of control)
Control (no treatment)	100	100	100	100
Pyrazofurin (10 mg/kg)	69†	82	66†	57
Pyrazofurin (10 mg/kg) and galactosamine (500 mg/kg)	12‡	21†	44†	129

* Means of three or more rats are given. Drugs were administered i.p. 21.5 hr before killing.
† Significantly different from values of untreated ($P < 0.05$).
‡ Significantly different from pyrazofurin-treated ($P < 0.05$).

Table 7. Effects of galactosamine, pyrazofurin and orotate on UDP, UTP and CTP concentrations in hepatoma 3924A and host liver*

Treatment	UDP		UTP		CTP	
	Host liver	Hepatoma 3924A	Host liver	Hepatoma 3924A	Host liver	Hepatoma 3924A
Control (no treatment)	100	100	100	100	100	100
Galactosamine (500 mg/kg)	86	87	25†	75	124	66†
Pyrazofurin (10 mg/kg)	82	55	75	50	78	57†
Galactosamine (500 mg/kg) and pyrazofurin (10 mg/kg)	24†	25†	7†	22†	145	48†
Galactosamine (500 mg/kg), pyrazofurin (10 mg/kg) and orotate (300 mg/kg)	7†	25†	5†	21†	123	45†

* Means of three or more rats are given. Drugs were given 17 and 2 hr before killing. Data are per cent of controls.

† Significantly different from untreated ($P < 0.05$).

single biochemical discriminant that enables them unequivocally to be distinguished from untransformed cells, the overall pattern of metabolic regulation in tumor cells shows many distinctive features [18, 19]. Thus, our approach to cancer chemotherapy is to attempt to devise drug combinations which will exert their greatest combined toxicity against cells with the enzyme pattern characteristic of neoplasia [1, 2]. The biochemical observations which prompted examination of the combinations of 3-deazauridine or pyrazofurin with D-galactosamine are: (a) the enzymes that metabolize $GalNH_2$ are most active in hepatic cells [20, 21], (b) the target enzyme for 3-deazauridine, CTP synthetase, shows neoplastic transformation linked increases in activity in hepatomas [22], and (c) the enzyme responsible for metabolic activation of pyrazofurin, adenosine kinase, is much more active in hepatomas of slow or medium growth rate than in such nonmalignant cell renewal compartments as bonemarrow, thymus, or intestinal epithelium. Thus, it was hoped that the combined toxicity of the combinations would be greatest in neoplastically transformed cells of hepatic origin.

The combination of $GalNH_2$ plus 3-DAU behaved as predicted in giving synergistic decreases in cellular CTP content, both *in vitro* and *in vivo* (Tables 1 and 2). As expected, uridine was able to prevent or reverse the $GalNH_2$-induced depletion of UTP, both in liver and in hepatoma (Tables 3 and 4). The effect of orotate was not as expected. Since orotate is able to enter liver cells readily, but its transport into hepatoma cells is impaired markedly, it was hoped that orotate could provide a selective rescue from $GalNH_2$ by reversing the UTP depletion in liver without any effect on hepatoma cells. This was not the case, as shown in Tables 4 and 5, since orotate increased the UTP content of hepatomas. A possible explanation for this anomaly is suggested by the data of Table 3, which indicate that orotate gave a large overshoot in the UTP content of normal rats; this result suggests that there is little or no feedback regulation of the *de novo* pyrimidine biosynthetic pathway of liver cells beyond the orotate phosphoribosyltransferase stage. The resulting large accumulation of UTP in the liver, to almost 5-fold the normal value, would probably be accompanied by a breakdown of UTP to uridine, which could enter the circulation and replenish the UTP pool of the tumor. The possibility remains that under appropriate conditions a selective rescue could be obtained with orotate, and this area is a subject for continuing research in this laboratory.

The possibility that hepatomas, by virtue of increased activity of the *de novo* pyrimidine pathway, could partly overcome the effect of $GalNH_2$ on UTP pools was suggested by Keppler [8], who showed that 6-azauridine could be an effective agent to combine with $GalNH_2$. In our studies, PF, which after metabolic activation acts at the same metabolic site as 6-azauridine, provided the additional advantage that it probably would be more rapidly activated in hepatomas than in bone marrow or intestinal epithelium, because of the higher adenosine kinase activity of hepatomas. The results did indeed show that PF and $GalNH_2$ provided an effective means of decreasing the pyrimidine nucleotide pools of hepatoma cells. That the combination had some degree of specificity toward the neoplastically transformed cells was clear from the fact that significant decreases were seen in the CTP content of hepatomas 8999 and 3924A, without any decrease in the host liver CTP. Pyrazofurin abolished the orotate rescue from $GalNH_2$ (Table 7). This was not unexpected, since when OMP decarboxylase is inhibited, orotate is unable to act as a source of uridine nucleotides.

Slowly growing solid tumors remain one of the most refractory problems in the chemotherapy of malignant disease. Our experiments represent an attempt to show how consideration of enzyme patterns of these tumors may suggest ways of combining antitumor drugs to optimize their response to treatment. The therapeutic properties of the combinations of (1) $GalNH_2$ and PF, and (2) $GalNH_2$ and 3-DAU are under current investigation in this laboratory.

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