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Selective Enhancement of 5-Fluorouridine Uptake and Action in Rat Hepatomas *in Vivo* Following Pretreatment with D-Galactosamine and 6-Azauridine or *N*-(Phosphonacetyl)-L-aspartate*

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Abstract—*The sequential combination of three antipyrimidines was studied in rats carrying Morris hepatoma 7777, the AS-30D ascites hepatoma, or the solid intrahepatic AS-30D tumor. The uptake of* [^{14}C] *5-fluorouridine and its incorporation into RNA was selectively enhanced in hepatomas* in vivo *by pretreatment of the animals with* D-*galactosamine and an inhibitor of* de novo *pyrimidine synthesis, such as 6-azauridine or* N-(*phosphonacetyl*)-L-*aspartate. This pretreatment resulted in a transient depletion of uridine phosphate pools which was the prerequisite for the subsequent increase in 5-fluorouridine phosphorylation in the hepatoma. It was demonstrated by radio-paper chromatography that the formation of 5-fluorouridine diphosphate* N-*acetylhexosamines was markedly enhanced when the pretreatment included* D-*galactosamine. The incorporation of labeled precursors into nucleic acids of AS-30D cells treated with 5-fluorouridine indicated that a severe inhibition of thymidylate synthase was associated with only a moderate depression of DNA synthesis, as measured by incorporation into DNA of* [^{3}H] *deoxyuridine and* [^{14}C] *deoxyadenosine, respectively. Survival of rats bearing the intrahepatic or the ascites form of the AS-30D hepatoma was prolonged most after the sequential treatment with* D-*galactosamine + 6-azauridine + 5-fluorouridine. When 6-azauridine was replaced in this combination by* N-(*phosphonacetyl*)-L-*aspartate, 80% of ascites hepatoma-bearing rats became tumor-free.*

INTRODUCTION

5-FLUOROURACIL (5-FUra) has been widely used in the chemotherapy of human hepatocellular carcinoma [1]. However, the effectiveness of 5-FUra as a single agent in this tumor has been unsatisfactory [2]. An early study by Heidelberger *et al.* [3] in the Novikoff hepatoma of rats has shown that the ribonucleoside 5-fluorouridine (5-FUrd) is much more effective than 5-FUra. The rate-determining step in the formation of the active phosphorylated metabolites of 5-FUrd is catalyzed by uridine kinase [4–7]. The activity of this enzyme can be raised by a cell-specific depletion of UTP and CTP [8, 9] that function as potent feedback inhibitors of uridine kinase [10]. Thereby an increased uptake of 5-FUrd, in contrast to 5-FUra, has been achieved in ascites hepatoma cell suspensions *in vitro* [8]. UTP pools can be lowered specifically in hepatoma cells and hepatocytes by the combination of uridylate trapping induced by D-galactosamine (GalN) and inhibition of *de novo* pyrimidine synthesis by 6-azauridine (6-AzaUrd) [11] or *N*-(phosphon-acetyl)-L-aspartate (PALA) [12] or pyrazofurin [13]. The transient effect of these antipyrimidines on UTP levels serves to specify the enhanced uptake of 5-FUrd rather than to damage hepatoma cells and hepatocytes. Chemotherapy of hepatomas solely by agents inducing UTP deficiency is complicated *in vivo* both by UTP synthesis on the salvage path-

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way and by liver damage that would result from a long-term depletion of UTP required for a lethal effect on hepatoma cells [11]. The chemotherapeutic effectiveness of 5-FUrd is considered to result from the inhibition of thymidylate synthase by FdUMP [7, 14] as well as from the incorporation of FUMP into RNA [7, 15, 16]. Among the fluorinated pyrimidines 5-FUrd is the most efficient precursor for RNA synthesis [17]. This analog incorporation is further enhanced when 5-FUrd is administered subsequent to a depletion of UTP pools in hepatomas *in vivo*. The sequential administration of GalN, an inhibitor of *de novo* pyrimidine synthesis, and 5-FUrd is more effective than 5-FUrd alone in the chemotherapy of hepatoma-bearing rats. It will be shown furthermore that the pretreatment enhances the selectivity of 5-FUrd for solid hepatomas.

MATERIALS AND METHODS

Rats and hepatoma lines

Female Sprague–Dawley rats (Voss, Tuttlingen, F.R.G.) were used for transplantation of AS-30D ascites hepatoma cells [18]. Transplantation and collection of AS-30D cells was performed as described earlier [19]; transplant generations 440–470 were used in the present study. Solid intrahepatic tumors were produced by injection of AS-30D cells directly into the liver under ether anesthesia. Solid AS-30D tumors with a diameter of more than 1 cm were seen after a period of about 10 days.

Morris hepatoma 7777, originally obtained from Dr. H. P. Morris, Washington, D.C., U.S.A., and kindly provided by Dr. W. Reutter, Freiburg, F.R.G., was transplanted bilaterally into the thigh muscle of female Buffalo rats. Generations 205–210 were studied 2 weeks after inoculation. The hepatoma was freeze-clamped *in situ* [20].

All rats were fed an unrestricted, 20% protein, carbohydrate-rich diet (Altromin, Lage, F.R.G.) and kept under controlled light cycle and room temperature.

Chemicals, enzymes and isotopes

D-Galactosamine · HCl (GalN) was purchased from C. Roth, Karlsruhe, F.R.G.; 6-azauridine (6-AzaUrd), 5-fluorouracil (5-FUra), 5-fluorouridine (5-FUrd), and uridine (Urd) were obtained from Calbiochem-Behring, La Jolla, Ca., U.S.A.. *N*-(phosphonacetyl)-L-aspartate (PALA), NSC 224131, was kindly supplied by the National Cancer Institute, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, Bethesda, Md., U.S.A.. Swim's S-77 powdered medium and Ham's medium F-12 were from Grand Island Biological Co., Grand Island, N.Y., U.S.A.. All coenzymes and enzymes required for nucleotide analyses were purchased from Boehringer Mannheim, Mannheim, F.R.G.

[2-^{14}C] 5-Fluorouridine (52 Ci/mole) was obtained from Moravek Biochemicals, City of Industry, Ca, U.S.A. The following labeled nucleosides were from Amersham Buchler, Braunschweig, F.R.G.: [U-^{14}C]deoxyadenosine (53 Ci/mole), [2-^{14}C]deoxythymidine (58 Ci/mole), [2-^{14}C]deoxyuridine (58 Ci/mole), [1′, 2′-^{3}H]deoxyuridine (42 Ci/mmole), and [U-^{14}C]guanosine (545 Ci/mole).

Incubation of ascites hepatoma cells

AS-30D cells were collected, washed and suspended as described earlier [19]; the standard medium for the incubation [19] was modified with respect to the final concentrations of phosphate (2 mmole/l) and sodium bicarbonate (25 mmole/l). Incubations were terminated by freezing the cell pellets [19].

RESULTS

Changes in uridine 5′-phosphate contents of hepatomas and livers induced by antipyrimidines

The levels of UDP-glucose, UTP and UDP were markedly lower in untreated Morris hepatoma 7777 as compared to the respective values in host liver (Table 1). These uracil nucleotide contents in the hepatoma were in close agreement with previous measurements (Reutter and Bauer, personal communication, 1978). GalN treatment resulted in a depletion of these uracil nucleotides only in liver but not in the hepatoma. The small dose of FUrd (15 μmole/kg) was insufficient to change the pyrimidine nucleotide levels significantly (Table 1), although fluorinated uracil nucleotides were included [8, 22, 23] in the enzymatic assay for uracil nucleotides [21, 24]. In the hepatoma, the synergistic action of GalN and an inhibitor of *de novo* pyrimidine synthesis (6-AzaUrd or PALA) was required for a marked reduction of UTP+UDP and UDP-glucose (Table 1).

The UTP deficiency in host liver was not induced when ascites hepatoma-bearing rats were injected i.p. with GalN+6-AzaUrd (Fig. 1). Under this condition, however, a depletion of uridine phosphates was observed selectively

Table 1. Uracil nucleotide contents in Morris hepatoma 7777 and host liver after administration of antipyrimidines (μmole/g wet wt)

Treatment	Morris hepatoma			Liver		
	UDPG	UTP+UDP	UMP	UDPG	UTP+UDP	UMP
None	0.10±0.01	0.10±0.03	0.11±0.06	0.26±0.12	0.21±0.03	0.10±0.03
5-FUrd	0.10±0.01	0.14±0.02	0.13±0.03	0.27±0.05	0.19±0.001	0.15±0.01
6-AzaUrd +GalN +5-FUrd	0.02±0.004	0.04±0.01	0.18±0.04	0.01±0.005	0.04±0.01	0.15±0.02
PALA +GalN +5-FUrd	0.03±0.01	0.05±0.01	0.15±0.02	0.01±0.002	0.03±0.01	0.12±0.01
GalN +FUrd	0.08±0.01	0.13±0.02	0.14±0.02	0.02±0.002	0.05±0.002	0.12±0.004

Female Buffalo rats bearing the Morris hepatoma 7777 bilaterally in the thigh muscle were starved overnight and subjected to treatment with antipyrimidines at doses of 1000, 120, and 200 μmole/kg body wt for GalN, 6-AzaUrd, and PALA, respectively. GalN and 6-AzaUrd were injected i.p. simultaneously, whereas PALA was given 3 hr before GalN. 5-FUrd (15 μmole/kg) was injected/i.v. 2 hr after GalN treatment. All animals were sacrificed under pentobarbital anesthesia (45 mg/kg) 2 hr after the dose of 5-FUrd. The livers and hepatomas were collected by freeze-clamp technique [20]. Uracil nucleotides were determined enzymatically [21]. This assay includes the small amount of 5-fluorouracil nucleotides. Each value represents the mean from 3 animals±S.D.; for the hepatoma $n=6$.

in the ascites hepatoma cells. This depletion was dose-dependent and corresponded to a reduction by 80% when measured 1 hr after treatment with GalN (300 μmole/kg) and 6-AzaUrd (120 μmole/kg) (Fig. 1). In host liver a strong rise in uracil nucleotide contents was elicited following the antipyrimidine injection into the ascites hepatoma (Fig. 1).

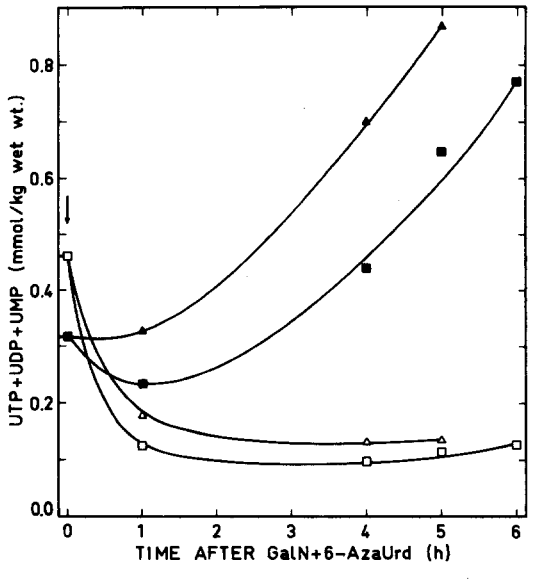

Fig. 1. Effect of GalN and 6-AzaUrd on the uridine 5'-phosphate content of hepatoma cells and liver in vivo. *Female Sprague–Dawley rats carrying the AS-30D ascites hepatoma for* 7 days *received i.p. injections of GalN* (300 μmole/kg)+6-*AzaUrd* (120 μmole/kg) *or, at a lower dose, GalN* (115 μmole/kg)+6-*AzaUrd* (40 μmole/kg) (*arrow*). *The uridine 5'-phosphate contents of AS-30D cells* (*open symbols*) *and liver* (*closed symbols*) *were determined enzymatically* [21]. *Each point represents the mean from* 3 *to* 6 *animals with the* S.D. $<10\%$ *of the mean. Triangles correspond to low-dose antipyrimidine treatment and squares represent the values after the high dose of GalN+6-AzaUrd.*

Uptake of [^{14}C] *5-fluorouridine after antipyrimidine pretreatment* in vivo

UTP deficiency has been used to increase the uptake of 5-FUrd by hepatoma cells *in vitro* [8]. A 2.2-fold rise in the uptake of 5-FUrd by the solid Morris hepatoma 7777 was demonstrated when the tumor-bearing rats had been pretreated with GalN+6-AzaUrd 2 hr before the [^{14}C]FUrd injection (Table 2). Incorporation of label into the tumor during the 2 hr period allowed for uptake was also enhanced when PALA and GalN had been administered in order to reduce the UTP level. Identification by radio paper chromatography of the nucleotides formed from [^{14}C]-5-FUrd in the hepatoma revealed the formation of 5-FUDP–hexosamines and the accumulation of 5-FUDP-*N*-acetylhexosamines when the pretreatment included GalN (Fig. 2).

Table 2. Distribution of radioactivity in tissues of rats bearing Morris hepatoma 7777 after administration of [^{14}C]5-*fluorouridine* (nCi/g *tissue wet wt*)

Tissue	Pretreatment None	6-AzaUrd + GalN	PALA + GalN	GalN
Hepatoma	15.3±3.7	33.6±7.6	24.1±4.9	19.2±1.9
Liver	19.3±4.7	18.2±5.3	17.5±2.4	18.1±0.9
Plasma	4.8±0.5	4.4±0.3	3.8±1.4	4.0±0.2
Spleen	12.5±2.8	16.9±2.8	21.1±3.8	20.9±3.0
Kidney	42.8±6.4	113.1±13.0	155.1±15.8	78.6±14.7
Intestine	19.4±1.3	24.7±6.3	33.4±16.3	22.6±0.5
Brain	6.9±0.8	7.3±0.4	6.4±0.7	9.2±0.6

Hepatoma-bearing Buffalo rats were treated as described in the legend to Table 1. [^{14}C]5-FUrd was injected i.v. at a specific activity of 6.7Ci/mole and at the same dose as given under Table 1. Heparinized blood was withdrawn from the aorta; all organs were rapidly removed and freeze-clamped. Tissue homogenates and plasma were counted in a triton–toluene-based liquid scintillation mixture at a counting efficiency of 90%. Each value represents the mean from 3 animals±S.D., for the hepatomas $n=6$.

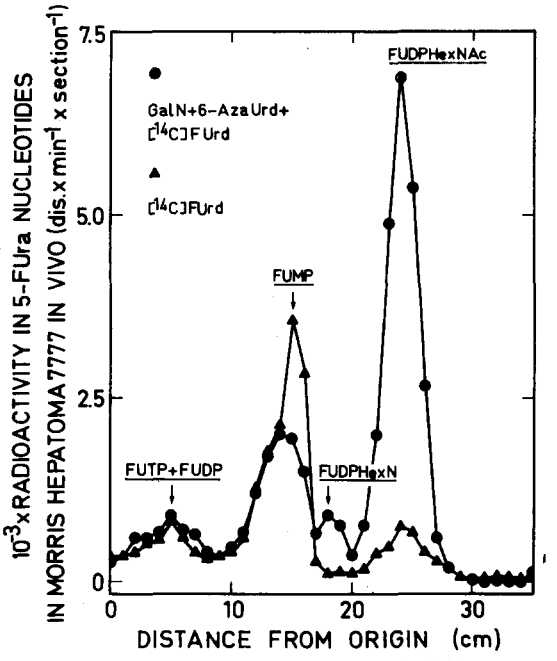

Fig. 2. Radio-paper chromatography of acid-soluble extracts of Morris hepatoma 7777 labeled with [^{14}C]5-*FUrd* in vivo. *The hepatoma-bearing rats were treated with GalN*+6-*AzaUrd and* [^{14}C]5-*FUrd as described in the legends to Tables* 1 *and* 2, *respectively. The acid-soluble supernatants were chromatographed on Whatman* 3 *MM paper with the solvent ethanol/ammonium acetate* (1 mole/l, pH 7.5) (5/2) *for* 40 hr. *The radioactivity in each* 1-cm *strip was determined in a toluene-based scintillation mixture with a* 70% *efficiency.*

These 5-FUDP-amino sugars provide a source for 5-FUMP, 5-FUDP, and 5-FUTP. 5-FUTP served as a substrate for RNA synthesis, and the incorporation of 5-FUMP into hepatoma RNA was increased 3.6-fold when the pretreatment consisted of GalN + 6-AzaUrd (Table 3). However, the antipyrimidine combinations did not enhance the incorporation of FUMP into host liver RNA, nor was the radioactivity in the acid-soluble fraction augmented significantly. The comparison of the uptake values of 5-FUrd in hepatoma and liver indicates that improved selectivity for the tumor can be achieved by the sequential combination of GalN and an inhibitor of *de novo* pyrimidine synthesis followed by the fluorinated uridine analog. It is of interest, however, that the highest labeling from [^{14}C]-5-FUrd was observed in kidney and that this radioactivity was augmented most after antipyrimidine pretreatment (Table 2).

Table 3. Distribution of radioactivity in Morris hepatoma 7777 and rat liver after administration of [^{14}C]5-*fluorouridine*

Pretreatment	Morris hepatoma 7777 Acid-soluble supernatant (nCi/g tumor wet wt.)	RNA (nCi/g RNA)	Host liver Acid-soluble supernatant (nCi/g liver wet wt.)	RNA (nCi/g RNA)
None	10±2	733±187	10±2	327±79
6-AzaUrd + GalN	19±4	2652±849	18±8	236±73
PALA±GalN	13±3	1815±387	14±2	268±25
GalN	12±1	954±133	13±0.1	215±54

Hepatoma-bearing Buffalo rats were treated with antipyrimidines and [^{14}C]5-FUrd as described under Tables 1 and 2. The tissues were homogenized in 5 vol of ice-cold perchloric acid (0.6 mole/l); acid-soluble supernatants were neutralized and counted for radioactivity; RNA was extracted and hydrolyzed from the acid precipitates and determined spectrophotometrically at 260 nm[25]. The amounts of RNA extracted from hepatoma and liver were 7.6 ±0.4 and 7.2±1.1 mg/g wet tissue±S.D., respectively. Radioactivity in the RNA hydrolysates was measured by liquid scintillation counting. Each value represents the mean±S.D. from 3 livers and 6 Morris hepatomas, respectively.

Effect of 5-fluorouridine and other antipyrimidines on the incorporation of precursors into nucleic acids

5-Fluorodeoxyuridylate (5-FdUMP) has been identified as a metabolite of 5-FUrd [17] and as a powerful inhibitor of thymidylate synthase [7, 14]. This inhibition of TMP synthesis was indicated *in vitro* and *in vivo* by the strong reduction of deoxyuridine (dUrd) incorporation into DNA (Fig. 3, Table 4). This effect on TMP synthase was markedly prolonged when 5-FUrd TMP synthase need not lead to a depletion of TTP pools under conditions of an active thymidine (dThd) phosphorylation on the salvage pathway [26]. The incorporation of [^{14}C]dAdo into DNA *in vivo* was affected much less by 5-FUrd when compared to the labeling from dUrd (Fig. 3B, Table 4). This indicates that the inhibition of TMP synthase was not followed by a proportional reduction of DNA synthesis. In hepatoma cell suspen-

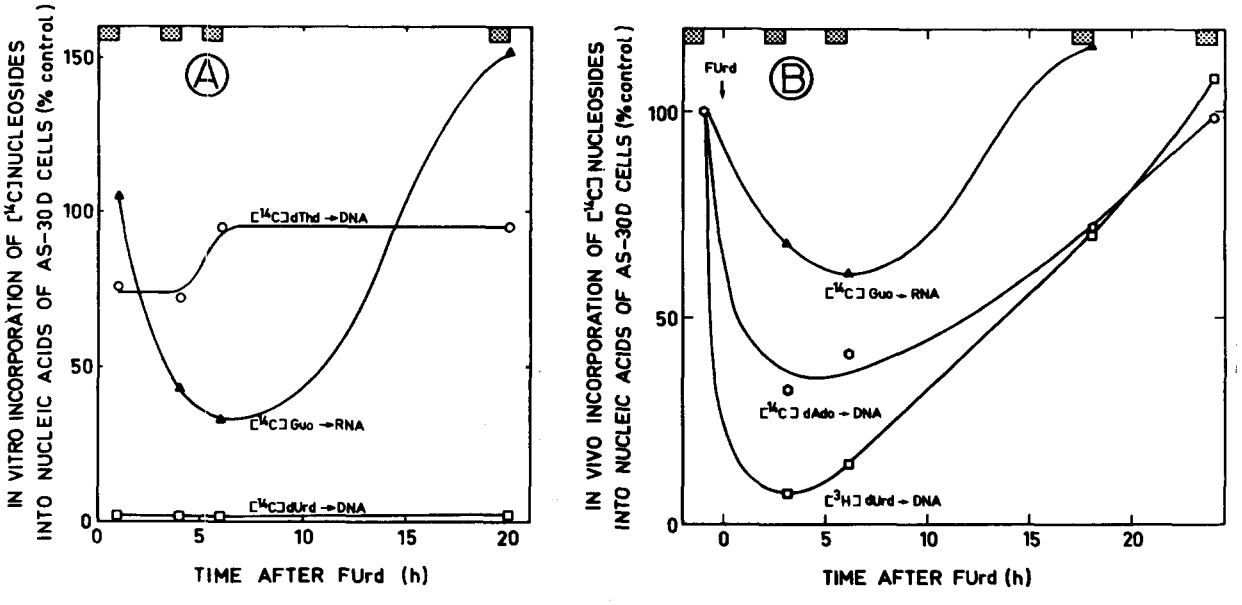

Fig. 3. Incorporation of labeled precursors into nucleic acids of hepatoma cells pretreated with antipyrimidines in vitro (*A*) *and* in vivo (*B*). (*A*) *AS-30D ascites hepatoma cells were incubated in the presence of GalN* (500 μmole/l) + 6-*AzaUrd* (200 μmole/l). 5-*FUrd* (230 μmole/l) *was added after* 180 min [8] *and the incubation was continued for another* 30 min *when the cells were harvested and reincubated in fresh medium devoid of antipyrimidines. The* 1-hr *incorporations of* [^{14}C]*dThd* (40 μCi/l), *and* [^{14}C]*dUrd* (40 μCi/l) *into DNA, and* [^{14}C]*Guo* (80 μCi/l) *into RNA, respectively, were determined at* 1, 4, 6, *and* 20 hr *after 5-FUrd. RNA was measured as described under Table* 3 [25]; *subsequently DNA was extracted and hydrolyzed with perchloric acid* (0.6 mole/l; 90°C *for* 1 hr). *Radioactivity in the nucleic acid hydrolysate was measured by liquid scintillation counting. The results are presented as the percentage of the incorporation in control cells never exposed to antipyrimidines. The absolute incorporation in untreated cells was* 240, 220, *and* 28 μCi/g *of nucleic acid for dThd, dUrd and Guo, respectively. Each point represents the mean from* 3 *experiments with the* S.D. < 12% *of the mean. The horizontal length of the dotted areas indicates the incubation period in the presence of the labeled precursors.* (*B*) *Female Sprague–Dawley rats were challenged with* 1 × 10 (± 10%) *of AS-30D ascites hepatoma cells i.p. Six days later, the animals received i.p. injections of antipyrimidines at doses of* 300, 120, *and* 10 μmole/kg *body weight for GalN,* 6-*AzaUrd, and* 5-*FUrd, respectively. *GalN and* 6-*AzaUrd were given simultaneously,* 1 hr *prior to* 5-*FUrd. The animals were sacrificed at* 4, 7, 19, *and* 25 hr *after treatment with GalN* + 6-*AzaUrd.* [^{14}C]*dAdo,* [^{3}H]*dUrd, and* [^{14}C]*Guo were injected i.p.* 1 hr *before sacrifice at doses of* 37.5, 100, *and* 50 μCi/kg, *respectively. Under ether anesthesia, the ascites fluid was rapidly removed and freeze-pellets were prepared* [19]. *Nucleic acids were extracted* [25] *and analyzed for radioactivity. The absolute incorporation in untreated control cells was* 1200, 80,000 and 7300 μCi/g *of nucleic acid for dAdo, dUrd and Guo, respectively. Each value represents the mean from* 3–9 *animals with the* S.D. < 20% *of the mean.*

was combined with GalN + 6-AzaUrd (Table 4). However, when 5-FUrd was omitted, GalN + 6-AzaUrd also led to a marked reduction of dUrd incorporation into DNA; this was most evident after 19 hr. At this time 5-FUrd alone was ineffective in this respect in the *in vivo* experiment (Table 4). The rate of DNA synthesis may be reflected more accurately by the incorporation of labeled deoxyadenosine (dAdo) since the inhibition of sions *in vitro*, the [^{14}C]dUrd incorporation into DNA remained for at least 20 hr below 1% of the control value that was obtained from cells incubated in the absence of antipyrimidines (Fig. 3A). This inhibition *in vitro* was observed to a similar extent whether or not GalN + 6-AzaUrd were combined with 5-FUrd. [^{14}C]dThd labeling of DNA, however, did not indicate a considerable decrease in the rate of DNA synthesis (Fig. 3A).

Table 4. Incorporation of labeled precursors into nucleic acids of AS-30D ascites hepatoma cells in vivo

Treatment	Incorporation period following the initial treatment (hr)	[^{14}C]dAdo →DNA	[^{3}H]dUrd →DNA	[^{14}C]Guo →RNA
		(percentage of incorporation in untreated cells)		
GalN+6-AzaUrd	3–4	32.7±8.8 (9)	7.6± 4.6 (9)	68.3±12.8 (3)
+5-FUrd	18–19	71.4±3.9 (3)	68.7±34.1 (3)	117.1±24.2 (3)
5-FUrd	3–4	55.5±9.7 (6)	8.7±3.4 (6)	—
	18–19	84.7±7.3 (3)	154.3±7.3 (3)	—
GalN+6-AzaUrd	3–4	46.1±6.9 (9)	46.2±25.2 (9)	71.3±2.6 (3)
	18–19	—	66.6±10.2 (3)	81.7±6.3 (3)
GalN+6-AzaUrd +Urd	3–4	89.0±11.1 (6)	—	—

Hepatoma-bearing rats were treated as described in the legend to Fig. 3B. Urd at the dose of 1000 μmole/kg was used in place of 5-FUrd in one group. Each value represents the mean±S.D. The number of animals in each group is shown in parenthesis.

Table 5. Survival of rats after transplantation of AS-30D ascites hepatoma cells and chemotherapy

Treatment	Exp. 1	Exp. 2
	(Median survival time (days) and 70-day survivors (in parenthesis; %)	
None	19.1 (6)	20.6 (0)
5-FUrd	26.1 (0)	29.1 (15)
6-AzaUrd+5-FUrd	—	37.7 (40)
GalN+6-AzaUrd	17.0 (6)	—
GalN+6-AzaUrd +5-FUrd	35.3 (29)	40.7 (35)
Number of rats in each group	17	20
Site of tumor implantation (1.4×10^6 cells per rat)	intrahepatic	i.p.
Doses (μmole/kg):		
5-FUrd	15	10
6-AzaUrd	120	40
GalN	300	300
Injection schedules	sequentially i.p. 10 min intervals; every second day; totally 5 times	i.p.; 5-FUrd 60 min after the other antipyrimidines; every third day; totally 20 times

Female Sprague–Dawley rats with initial body weights of 51±3 and 83±3 g ($\bar{x}$±S.D.) were used in experiments 1 and 2, respectively. The chemotherapy was started 1 day after tumor cell transplantation. The animals were kept under close surveillance for the period of 70 days. The median survival time during this 70-day period and the percentage of 70-day survivors are given.

UTP deficiency induced by GalN+6-AzaUrd in the AS-30D cells (Fig. 1) resulted in a transient depression of [^{14}C]guanosine (Guo) incorporation into RNA (Table 4). These antipyrimidines caused an even stronger inhibition of DNA synthesis that was measured by labeling with [^{14}C]dAdo. This effect on DNA synthesis could be reverted by uridine administration (Table 4).

Chemotherapeutic effects of sequentially administered antipyrimidines in hepatoma-bearing rats

Induction of UTP deficiency by GalN+6-AzaUrd in AS-30D hepatoma cells *in vivo* did not result in a significant prolongation of the survival time of the rats (Table 5). The antipyrimidine dosage used was insufficient to produce long-term and thereby lethal UTP deficiency in these tumor cells. The transient reduction of UTP pools, however, served effectively to enhance the effect of 5-FUrd on the survival of rats bearing intrahepatic solid AS-30D hepatomas that were usually accompanied by the ascitic form of this tumor. Only the sequential administration of the three antipyrimidines was followed by a survival of 29% of the animals for more than 70 days, although 5-FUrd alone had some inhibitory effect on the growth of this tumor at the dose of 15 μmole/kg (Table 5, exp. 1), 6-AzaUrd injected in a 4-fold molar excess 60 min before 5-FUrd enhanced the effect of the latter analog to a similar extent as in the combination including GalN (Table 5, exp. 2). This result obtained with the ascites hepatoma

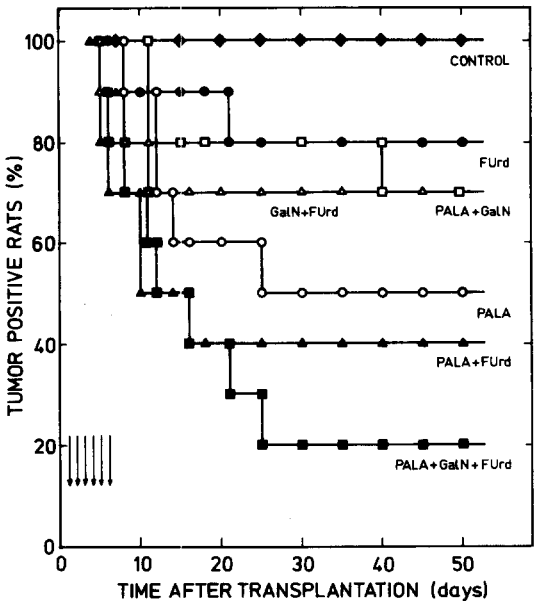

Fig. 4. Enhancement of 5-fluorouridine action on AS-30D ascites hepatoma by pretreatment with N-(*phosphonacetyl*)-L-*aspartate and* D-*galactosamine. Female Sprague–Dawley rats with an initial body weight of* 67 ± 3 g *were transplanted i.p. with* 1.3×10^6 *AS-30D cells suspended in Ham's F-12 medium* (0.2 ml). *The antipyrimidine treatment was initiated* 1 day *after tumor transplantation and comprised* 6 *doses, on* 6 *subsequent days as indicated by arrows, of 5-FUrd* (8 μmole/kg), *GalN* (500 μmole/kg), *and/or PALA* (200 μmole/kg). *PALA and GalN were injected simultaneously and followed by 5-FUrd after a* 60 min *time interval. Each group consisted of* 10 *rats.*

injected i.p. cannot be transferred to solid heptomas where GalN was required in combination with 6-AzaUrd for an effective lowering of UTP pools prior to 5-FUrd administration. GalN was a component, however, of the most effective antipyrimidine combination when 6-AzaUrd had been replaced by PALA as the inhibitor of *de novo* pyrimidine synthesis (Fig. 4). This treatment cured 80% of the tumor-bearing rats. Sixty per cent of the animals survived when FUrd administration was preceeded by a dose of PALA; GalN + 5-FUrd was less effective in eradicating the tumor (Fig. 4). The *in vivo* effectiveness of these combinations correlated with 5-FUrd uptake and incorporation into RNA by AS-30D hepatoma cells in suspension [8].

DISCUSSION

Drug selectivity for malignant tissues is a key problem in the chemotherapy of cancer. The selective toxicity can be enhanced by a combination of several antimetabolites. A cell-specific depletion of UTP pools in liver [27] and hepatocyte-derived tumors can be achieved by GalN together with an inhibitor of *de novo* pyrimidine synthesis [11, 12, 28]. Addition of the latter is required only in hepatomas [13, 23]. This UTP deficiency may serve as a 'specifier' for the uptake of pyrimidine ribonucleoside analogs [23] leading to an increased conversion of FUrd to its active phosphorylated metabolites [8]. An enhanced uptake of 5-azacytidine has been described in tumor cell suspensions pretreated with pyrazofurin as the inhibitor of *de novo* pyrimidine synthesis [29, 30]. *In vivo*, however, inhibition of *de novo* uridylate synthesis is insufficient to deplete UTP pools in most tissues [13, 23, 31]. We have therefore combined this inhibition with a trapping of uridylate by GalN (Fig. 1, Table 1). The resulting UTP deficiency can be short-lived *in vivo* if the antipyrimidine doses are low and if uridylate is provided on the salvage pathway. Under this condition, cytotoxicity and cell kill are still limited [11, 28] but the subsequent uptake of 5-FUrd can already be enhanced (Tables 2 and 3) [8]. Accordingly, the chemotherapeutic effectiveness of drug combinations such as GalN + 6-AzaUrd or GalN + PALA was negligible (Table 5) or less pronounced (Fig. 4) as compared to a sequential treatment including 5-FUrd (Table 5, Fig. 4). The pretreatment with antipyrimidines lowering UTP did not only increase the uptake of 5-FUrd [8] but also enhance its selectivity for the hepatoma as compared to liver (Tables 2 and 3). This refers to the 5-FUrd metabolites in the acid-soluble fraction as well as to the incorporation of 5-FUMP into RNA (Table 3).

Hepatotoxicity can result from treatment with agents depleting UTP [27]. This depends, however, on the size and location of the hepatoma in the animal as well as on the route of administration and dosage of the drug. Intraperitoneal injection of GalN + 6-AzaUrd in ascites hepatoma-bearing rats lowered the uridine phosphate contents in hepatoma cells but not in liver (Fig. 1). There was even a remarkable increase in hepatic uridine phosphates, probably caused by an enhanced availability of orotate produced by the hepatoma cells under the condition of a high rate of *de novo* pyrimidine synthesis and blocked orotidylate decarboxylase. On the other hand, hepatic UTP deficiency was detectable when rats were treated i.p. and when the hepatoma was located in the thigh muscle (Table 1). A severe depletion of rat liver UTP is tolerated without irreversible damage for up

to 3 hr [27]; only if this time period is exceeded, a rescue with pyrimidine nucleotide precursors, such as uridine or orotate [27], should be considered in order to counteract hepatotoxicity.

The use of 5-FUrd and other pyrimidine ribonucleoside analogs is favoured by the high activity of uridine kinase in hepatomas relative to liver [32, 33]. Moreover, uridine kinase activity can be enhanced by a lowering of its feedback inhibitors UTP and CTP in the hepatoma, the latter being the stronger inhibitor of this enzyme [10]. UTP deficiency is followed by a reduction of CTP pools in hepatoma cells *in vitro* [9] and *in vivo* [13] whereas the hepatic CTP content is increased under this condition [9, 34]. This differential response to pretreatment may explain the stimulation of 5-FUrd uptake in the hepatoma in contrast to liver (Table 2).

Incorporation of 5-FUMP into RNA may be more important for the chemotherapeutic effectiveness of fluorinated pyrimidines than the inhibition of thymidylate synthase by 5-FdUMP [15, 16]. The incorporation of dUrd into DNA reflects the inhibition of dUMP methylation rather than a true inhibition of DNA synthesis. The latter was much less depressed by 5-FUrd, when measured by labeling with dAdo, dThd (Fig. 3), or by incorporation into DNA of other precursors that are not directly affected by an inhibition of thymidylate synthase [7, 15, 35]. Blockade of TMP synthesis will not necessarily lead to a reduction of TTP pools sufficient for a decrease in the rate of DNA synthesis; this is most important *in vivo* in the presence of extracellular dThd [36] providing a source for intracellular TTP [26]. It cannot be decided at present whether the incorporation of 5-FUMP into RNA is the major site of action of 5-FUrd in the chemotherapy of hepatoma cells. In addition to the inhibition of TMP synthase, an interference of the 5-FUDP–amino sugars (Fig. 2) [8] in glycosylation reactions could be envisaged as another site of action of fluorinated pyrimidines.

The chemotherapeutic effectiveness of 5-FUrd could be enhanced best (Fig. 4) when its conversion to the active phosphorylated metabolites was most increased by prior administration of UTP-depleting antipyrimidines [8, 37]. GalN as a component of this drug combination improved the selectivity for hepatoma cells (Table 2) [23]. Our sequential combination chemotherapy may serve as a useful approach to the treatment of hepatocellular carcinoma.

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