INCREASED FORMATION OF NUCLEOTIDE DERIVATIVES OF 5-FLUOROURIDINE IN HEPATOMA CELLS TREATED WITH INHIBITORS OF PYRIMIDINE SYNTHESIS AND D-GALACTOSAMINE

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1. Introduction

Fluorinated pyrimidines and their nucleosides are widely used in the chemotherapy of tumors; their mode of action requires the formation of nucleotide derivatives (summary [1]). Uridine kinase (EC 2.7.1.48) plays a key role in the conversion of 5-fluorouridine (5-FUrd) to its active metabolites. The feedback regulation of uridine kinase by UTP [2] can be used for an increase in the cell-specific uptake of some pyrimidine analogs after depletion of the cellular UTP pools [3]. Induction of selective UTP deficiency by D-galactosamine (GalN) and 6-azauridine (6-aza-Urd) in hepatoma cells [4] is associated with an increased formation of phosphorylated derivatives of 5-FUrd [3]. This approach [3] has the following implications:

(i) Considerable selectivity for hepatoma cells and hepatocytes is based on the action of GalN [3,5];
(ii) The increased activity of uridine kinase [6,7] and the high rate of de novo pyrimidine nucleotide synthesis in hepatoma cells relative to liver [7–10] makes these malignant cells more susceptible to the action of 6-aza-Urd.
(iii) The formation and accumulation of 5-fluoro-UDP-amino sugars may serve as a metabolic store providing 5-FUrd phosphates; the latter become available in amino sugar transferase reactions or upon hydrolytic splitting of the fluorinated amino sugar nucleotide.

In this communication we demonstrate the negative correlation between 5-FUrd uptake and intracellular UTP level. The replenishment of depleted UTP pools with 5-fluoro-UTP (FUTP) is associated with an increased incorporation of 5-fluorouridylate into RNA. The analysis of 5-FUrd metabolites formed in the presence of GalN has revealed for the first time a formation and accumulation of 5-fluoro-UDP-hexosamines and 5-fluoro-UDP-*N*-acetylhexosamines.

2. Materials and methods

2.1. *Chemicals and enzymes*

D-Galactosamine·HCl (GalN) was purchased from C. Roth, Karlsruhe. 5-Fluorouridine (5-FUrd) and 6-azauridine (6-aza-Urd) were obtained from Calbiochem, San Diego. *N*-(Phosphonacetyl)-L-aspartate (PALA) was a generous gift from Dr G. Stark, Department of Biochemistry, School of Medicine, Stanford University. 5-Fluoro-[2-^{14}C]orotic acid, from ICN Pharmaceut., Irvine, served as precursor in the enzymatic synthesis of 5-fluoro-[2-^{14}C]uridylate [11]; 5-F[2-^{14}C]Urd was prepared by subsequent treatment with alkaline phosphatase and isolated by paper chromatography in a final purity of >99%. The other substrates and enzymes were from Boehringer Mannheim, Mannheim.

2.2. *Incubation of ascites hepatoma cells*

The transplantable ascites hepatoma AS-30D [12] was carried in female Sprague-Dawley rats. Transplant generations 500–530 were used in the present study. The tumor cells were collected and incubated as in [13] except phosphate and sodium bicarbonate in

the medium that were changed to 2 mmol/l and 25 mmol/l, respectively. Initial antipyrimidines in the incubation medium were 0.5 mmol/l for GalN, 0.2 mmol/l for 6-aza-Urd, 0.8 mmol/l for PALA, and 0.23 mmol/l for 5-FUrd. In experiments with 5-F[2-^{14}C]Urd spec. act. 1.2 Ci/mol was employed.

2.3. *Measurement of 5-fluoro-[^{14}C]uridine uptake and paper chromatography of its nucleotide derivatives*

GalN, 6-aza-Urd, PALA or combinations of these compounds were included in the suspension after a 40 min preincubation of the cells. Incubation in the presence of these inhibitors had lasted for 180 min when 5-F[2-^{14}C]Urd was added. 5-F[^{14}C]Urd uptake was terminated by mixing of 1 ml radioactive cell suspension with 10 ml unlabeled AS-30D cell suspension containing uridine (10 mmol/l) and kept at 4°C. Cell pellets were obtained without delay by centrifugation at 4°C and subsequently dissolved in a liquid scintillation mixture composed of Triton X-100, an equal vol. toluene, POPOP (200 mg/ml), and PPO (2 g/l). Counting efficiency was 90%.

Acid-soluble supernatants were obtained after freezing of the cells as in [13]. Supernatants from cells labeled for 90 min in the presence of 5-F[2-^{14}C]-Urd were spotted on Whatman 3 MM paper and chromatographed with the solvent ethanol/ammonium acetate (1 mol/l, pH 7.5) (5/2). ^{14}C-Labeled metabolites were localized by cutting 1 cm sections of the paper strips perpendicular to the direction of migration and counting at 70% efficiency in 10 ml toluene liquid scintillation mixture.

3. Results

3.1. *Influence of UTP level on the phosphorylation of 5-fluoro-uridine in hepatoma cells*

Different UTP levels in ascites hepatoma cells were adjusted by incubation of the cells in the presence of: (a) GalN that lowers the UTP concentration by its uridylate trapping action [4,5]; (b) PALA, a powerful inhibitor of aspartate carbamoyltransferase [14,15], causing a depression of cellular UTP levels in vitro [15,16]; (c) 6-aza-Urd that leads to an inhibition or orotidylate decarboxylase [17] and lowers the cellular UTP content in vitro [4]; (d) GalN

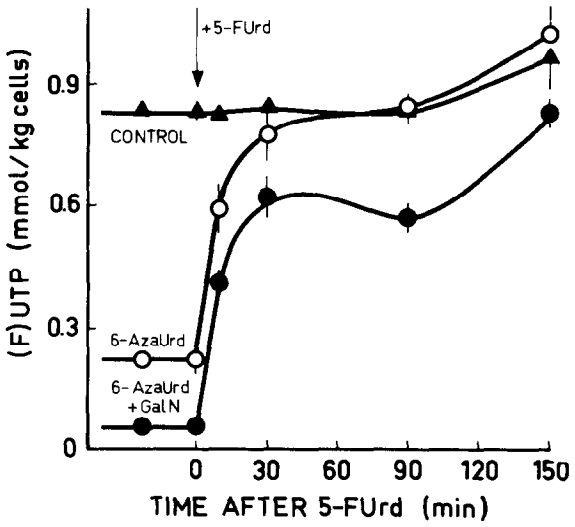

Fig.1. Contents of UTP and FUTP in hepatoma cells. AS-30D cells were incubated as in section 2.2. The preincubation in the presence of 6-aza-Urd (0.2 mmol/l), alone or in combination with GalN (0.5 mmol/l), lasted for 180 min when 5-FUrd (0.23 mmol/l) was added. The sum of UTP + FUTP ((F)UTP) was measured enzymatically [21]; 5-fluorouridine 5'-phosphates react, although at a lower rate, in this enzymatic sequence. The increase induced by 5-FUrd reflects exclusively FUTP in cells in which de novo pyrimidine synthesis has been blocked by 6-aza-Urd. Mean values from 4 experiments, vertical bars indicate SEM.

together with PALA [16] or 6-aza-Urd [4,10], both combinations leading to a depletion of UTP pools in vitro and in vivo. Incubation of AS-30D cells in the presence of these inhibitors for 3 h as in section 2.2. resulted in the following average UTP contents relative to control cells (100%): GalN, 60%; PALA, 45%; 6-aza-Urd, 24%; GalN + PALA, 7%; GalN + 6-aza-Urd, 5% (fig.1) [4,16]. The corresponding factors of increase in uptake of labeled 5-FUrd during 15 min relative to control cells (1.0) were 1.5 in cells treated with GalN, 2.5 with 6-aza-Urd, 3.1 with PALA, 3.3 with GalN + 6-aza-Urd, and 4.3 with GalN + PALA (fig.2).

Paper chromatography of the acid-soluble metabolites of 5-F[^{14}C]Urd indicated that >93% were phosphorylated derivatives when the UTP pool had been reduced below 10% before the addition of 5-FUrd. Induction of selective UTP deficiency [4] followed by addition of 5-FUrd resulted in a replenishment of the cellular UTP pool with FUTP (fig.1).

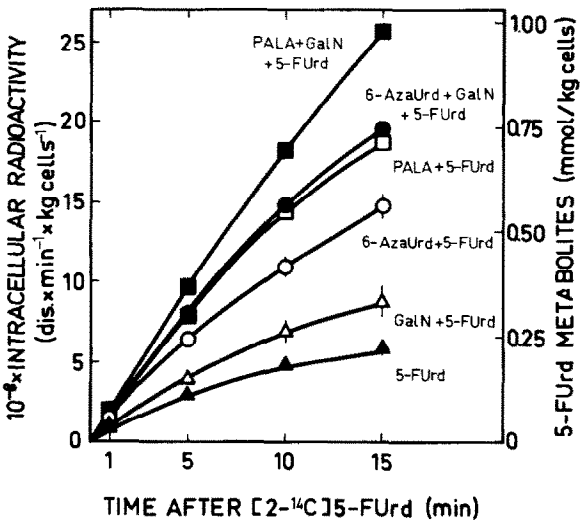

Fig.2. Uptake of 5-fluoro-[^{14}C]uridine into hepatoma cells pretreated with antipyrimidines. AS-30D cells were incubated at 37°C at 1.8–2.3 × 10^9 cells/l. The pH was kept between 7.45 and 7.25. For further details see sections 2.2. and 2.3. The radioactivities per sample ranged from 500–18 000 cpm. Mean values obtained from 6 experiments, vertical bars indicate ± SEM that was consistently <11% of the mean.

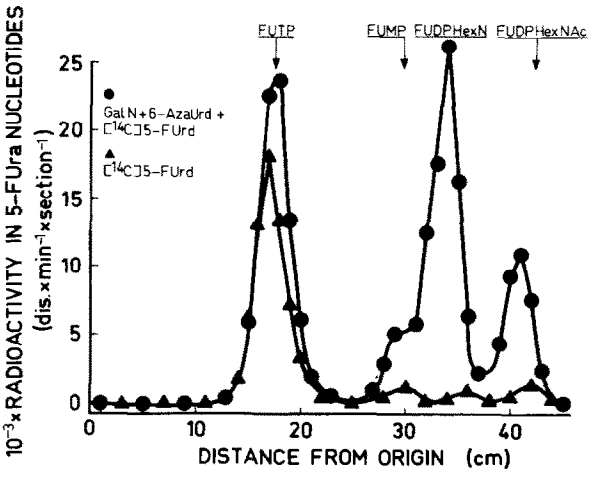

Fig.3. Radio-paper chromatography of acid-soluble extracts of AS-30D cells labeled with 5-F[^{14}C]Urd. Cells were incubated and treated as in section 2.2. and 2.3. Equal sample volumes, corresponding to 36 mg cells, were spotted on the paper. The pattern of fluorouracil nucleotides obtained after GalN treatment, in the presence or absence of 6-aza-Urd or PALA, was characterized by an accumulation of FUDP-HexN and a substantial increase in FUDP-HexNAc. Similar nucleotide patterns were observed in 4 different experiments performed under the same conditions.

However, the level of FUTP + UTP remained lower in the presence of GalN (fig.1). This contributes to an increased uptake of 5-FUrd into the cells since FUTP serves as feedback inhibitor of uridine kinase as well [2]. PALA supported the uptake of 5-FUrd better than 6-aza-Urd (fig.2). This was not due to a smaller depression of the UTP content by the 6-aza analog of uridine but may be related to an interference of 6-aza-Urd or its metabolite 6-aza-UMP with transport or phosphorylation of 5-FUrd.

3.2. *Formation of 5-fluorouridine diphosphate amino sugars in the presence of D-galactosamine*

The formation [18] and accumulation [19] of UDP-galactosamine and UDP-glucosamine (UDP-HexN) and of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine (UDPHexNAc) as metabolites of GalN has been described in liver and hepatoma cells [4,5,10]. Labelling of these amino sugar nucleotides with 5-F[2-^{14}C]Urd has now established the formation and accumulation of FUDP-HexN and FUDP-HexNAc (fig.3). Both fractions could also be labeled from [^{14}C]GalN and comigrated upon paper chromatography with UDP-[^{14}C]GalN and UDP-[^{14}C]GalNAc, respectively. Both fractions were resistant to treatment with alkaline phosphatase and yielded [^{14}C]FUMP upon hydrolysis with snake venom phosphodiesterase; the FUMP formed was assayed enzymatically [20]. Acid hydrolysis in HCl (0.1 mol/l) for 20 min at 95°C liberated [^{14}C]FUDP (80%) and [^{14}C]FUMP (20%) from FUDP-HexNAc but was insufficient to hydrolyze FUDP-HexN. The amount of FUDP-HexN formed during 90 min in the presence of GalN and 5-F[^{14}C]Urd was consistently higher than that of FUDP-HexNAc (fig.3). The combination of GalN + 6-aza-Urd + 5-FUrd resulted in a formation of FUDP-HexN and FUDP-HexNAc corresponding to ~38% (1.0 mmol/kg cells) and 16% (0.42 mmol/kg cells) of the total acid-soluble nucleotides, respectively (fig.3).

3.3. *Intracellular contents of 5-fluorouridine phosphates and incorporation into RNA*

Levels of FUTP of more than 0.5 mmol/kg cells, far in excess over the endogeneous UTP content, were

Table 1
Incorporation of 5-fluoro-[^{14}C]uridine

Antipyrimidines	Total cellular incorporation (mmol/kg)	Acid-soluble fraction (mmol/kg)	% total	[^{14}C]FUMP in RNA (mmol/kg)	% total
5-FUrd	0.92 ± 0.06	0.80 ± 0.03[a]	87.7	0.09 ± 0.04	9.6
6-Aza-Urd + 5-FUrd	1.60 ± 0.66	1.25 ± 0.56[b]	78.1	0.25 ± 0.03	15.5
GalN + 5-FUrd	1.48 ± 0.41	1.28 ± 0.34	86.4	0.20 ± 0.07	13.6
GalN + 6-Aza-Urd + 5-FUrd	2.95 ± 0.55	2.65 ± 0.46[c]	89.8	0.30 ± 0.12	10.2
PALA + 5-FUrd	1.57 ± 0.34	1.35 ± 0.31	85.6	0.23 ± 0.04	14.4
GalN + PALA + 5-FUrd	3.96 ± 0.43	3.60 ± 0.35	91.1	0.35 ± 0.09	9.0

[a,b,c] Total acid soluble 5-FUra 5′-nucleotides determined by enzymatic analysis [20] (mmol/kg cells ± SD): [a] 0.85 ± 0.06; [b] 1.48 ± 0.23; [c] 2.53 ± 0.13

AS-30D cells were incubated at 1.5–2.5 × 10^9/l in the presence of antipyrimidines as in section 2. Cells had been exposed, except for controls, to 6-aza-Urd, GalN, PALA or combinations of these for 180 min when 5-F[2-^{14}C]Urd was added for additional 90 min. RNA was hydrolyzed by treating the washed acid-insoluble fraction with NaOH (0.2 mol/l) for 20 h at 37°C and counted for radioactivity in fluoro-[^{14}C]uridylate. Incorporation as mmol/kg wet cells was calculated from the known specific activity of 5-F[^{14}C]Urd, from the radioactivity counted in the various fractions (700–9000 cpm), and from the cell wet weight determined [13]. Mean values from 3 different experiments ± SD are given

reached when 5-FUrd was added to cells depleted of UTP (fig.1). This replenishment of the UTP pool with FUTP was most rapid during the initial 10 min and almost complete after 30 min. Two major factors contributed to an increased incorporation of 5-FUrd into the cells: a low level of UTP and the continued trapping of fluorouridylate by GalN. The highest contents of 5-F[^{14}C]Urd metabolites were reached when an inhibitor of de novo pyrimidine synthesis and GalN were combined. This result was confirmed by enzymatic analysis (table 1). After 90 min, the uptake into the acid-soluble fraction was increased 4.5-fold to a level of 3.60 ± 0.35 (SD) mmol/kg cells when the cells had been pretreated with GalN + PALA (table 1). Under this condition 94% were phosphorylated derivatives of 5-FUrd and the content of FUTP amounted to 0.79 mmol/kg. The higher uptakes of 5-FUrd into the cells were paralleled by an increased incorporation of fluorouridylate in RNA (table 1).

4. Discussion

5-FUrd is one of the substrates of uridine kinase [22]. This enzyme is under feedback control by UTP and other pyrimidine nucleoside 5′-triphosphates including FUTP [2]. Induction of selective UTP deficiency and adjustment of different UTP levels in ascites hepatoma cells [4,16] led us to demonstrate the inverse relationship between 5-F[^{14}C]Urd uptake and UTP concentration in intact cells (fig.2). This result is consistent with the view that phosphorylation rather than transport of nucleosides is the rate-determining step in their incorporation into the cellular nucleotide pool [23]. The demonstration of FUDP-amino sugar formation and accumulation in cells treated with GalN and 5-FUrd (fig.2) serves to understand the promoting effect of GalN on 5-FUrd uptake (fig.3, table 1). Elevated levels of UDP-amino sugars are retained in the liver for many hours [19]. This indicates a slow regeneration of the UDP moiety. Accordingly, FUDP-amino sugars may serve as a metabolic store of FUDP. The latter is the precursor of both, FdUMP, a powerful inhibitor of thymidylate synthetase, and FUTP that is incorporated into RNA and interferes with processing and function of RNA (summary [1]). It is known at present whether FUDP-amino sugars are utilized by amino sugar transferases as substrates without interfering in the reaction. In *E. coli* K-12, 5-fluorouracil produces an osmotically sensitive state associated with an inhibition of cell-wall synthesis and an accumulation of *N*-acetylhexosamine esters [24]. A

similar phenomenon, however, has not been observed in mammalian cells treated with fluorinated pyrimidines.

Incorporation of 5-fluorouridylate into RNA was augmented in an additive manner when an inhibitor of de novo pyrimidine nucleotide synthesis was combined with GalN (table 1). This incorporation into RNA, under some conditions, appears as the key to the toxicity observed in Novikoff hepatoma cells in vitro [25] and to the chemotherapeutic effectiveness in experimental cancer in mice [26]. On the other hand, tissue retention of FdUMP and the associated inhibition of DNA synthesis appears as a major determinant in the therapeutic activity of 5-fluorouracil [27].

The sequential administration of GalN, 6-aza-Urd (or PALA), and 5-FUrd has been proposed as a protocol for the treatment of hepatocellular cancer [3,28]. A high degree of cell specificity is provided in this combination by GalN [3]. The accumulation of FUDP-amino sugars may prolong the action of 5-FUrd and reduce the extrahepatic toxicity.

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