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Separation of Several 5-Fluorouracil Metabolites in Various Melanoma Cell Lines. Evidence for the Synthesis of 5-Fluorouracil-Nucleotide Sugars*

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Abstract—*5-Fluorouracil (5FU) metabolism was studied in intact cancer cells kept in suspension by incubation with tritiated 5FU. Metabolites were analyzed using various chromatographic procedures, including a one-directional separation on PEI-cellulose sheets, which separated 5FU from the mono-, di- and triphosphate forms and from nucleotide sugars. The monophosphate ester was present as FdUMP, as could be demonstrated with another chromatographic procedure. In the human melanoma cell lines IGR3 and M5 the main metabolite of 5FU was 5-fluorouridine, while in the murine B16 melanoma only a small amount of 5-fluorouridine was formed. In B16 cells more 5FU label was present as the triphosphate ester while in M5 cells more FdUMP was formed. With all three cell lines 5FU was incorporated into RNA; this incorporation was stimulated by* 1 mM *N-(phosphonacetyl)-L-aspartate (PALA). PALA did not significantly affect the conversion of 5FU into other metabolites, nor did it affect the incorporation of 5FU into DNA. A 5FU-nucleotide sugar, present as the diphosphate-glucose, was a predominant 5FU-metabolite in M5 cells but not in the other cell lines. Its identity was confirmed by thin-layer chromatography and high-performance liquid chromatography. Its possible function is discussed.*

INTRODUCTION

THE PYRIMIDINE analog 5FU requires anabolism to nucleotides to show antitumor activity that is mediated via inhibition of DNA synthesis and/or incorporation of 5FU into RNA. In the presence of the cofactor 5,10-methylene tetrahydrofolate, a stable complex of TMP-synthetase–FdUMP-folate is formed [1], resulting in inhibition of synthesis of TMP from dUMP, leading to TTP depletion and inhibition of DNA synthesis. FdUMP can also be phosphorylated to FdUTP but hydrolysis catalyzed by dUTP nucleotide hydrolase prevents incorporation of FdUTP into DNA [2]. Uracil-DNA glycosylase removes FdUTP that occasionally will be incorporated into DNA [3, 4]. Nonetheless, some incorporation of fluoropyrimidines into DNA has been reported [5, 6]. 5-Fluorouracil is incorporated via FUTP into various species of RNA, including polysomal RNA, nuclear RNA and messenger RNA [7–9].

In vivo uridine metabolites are present to a large extent as nucleotide-sugars, such as UDP-glc. The synthesis of 5FU-nucleotide sugars received only minor attention. Pogolotti *et al.* [10] and Iigo *et al.* [11], using HPLC, reported the incorporation of 5FU into 5FU-nucleotide-hexoses, while Holstege *et al.* [12] demonstrated the synthesis of 5FU-nucleotide-hexosamines in

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Abbreviations: 5FU, 5-fluorouracil; FUR, 5-fluorouridine; FUMP, 5-fluorouridine-5'-monophosphate; FdUMP, 5-fluoro-2'deoxyuridine-5'-monophosphate; FUDP, 5-fluorouridine-5'-diphosphate; FUTP, 5-fluorouridine-5'-triphosphate; FdUTP, 5-fluoro-2'-deoxyuridine-5'-triphosphate; FUDP-glc, 5-fluorouridine-5'-diphosphate glucose; UDP-glc, uridine-5'-diphosphate glucose; PALA, *N*-(phosphonacetyl)-L-aspartate; HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography; PEI-cellulose, polyethyleneimine-cellulose.

the presence of galactosamine as a co-substrate. Detection of nucleotide-sugars is complicated since in various frequently used chromatographic systems co-elution with other metabolites occurs. Therefore we developed a number of procedures which separated nucleotide-sugars from other 5FU-metabolites and could demonstrate that synthesis of FUDP-sugars differed markedly in various melanoma cell lines. These methods also allow the separation of the other 5FU-metabolites from each other.

In our studies on modulation of pyrimidine metabolism in cancer cells we examined the effect of PALA, a potent inhibitor of aspartate transcarbamylase [13]. Treatment of cells with PALA caused depletion of UTP levels [14–16], which can enhance 5FU incorporation into RNA. In this paper we also describe the effect of PALA on the synthesis of several 5FU metabolites using diverse chromatographic methods. These studies were performed in various cell lines which show pronounced differences in pyrimidine metabolism; the murine melanoma B16 cell line has a very low uridine phosphorylase activity, while the human melanoma cell lines M5 and IGR3 have a much higher activity [17]. The data show that a nucleotide-sugar, FUDP-glc, may be an important metabolite only in certain cell lines, while the modulation by PALA on pyrimidine metabolism differs between the various cell lines.

MATERIALS AND METHODS

Materials

5-Fluorouracil and FdUMP were obtained from Sigma, St Louis, MO, U.S.A. and FUR and FUMP from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. [6-^{3}H]5-fluorouracil was from the Radiochemical Centre, Amersham, U.K., while Instagel, Dimilume and Soluene-350 were from Packard, Groningen, The Netherlands. Plastic sheets precoated with 0.1 mm PEI-cellulose were purchased from Merck, Darmstadt, F.R.G. Dulbecco's minimum essential medium and fetal bovine serum were purchased from GIBCO Europe Limited, Paisley, U.K. The sources of the B16, IGR3 and M5 cells have been reported previously [14].

Cell culture

Cells were cultured in Dulbecco's medium supplemented with 15% dialyzed, heat-inactivated (30 min, 56°C) fetal bovine serum at 37°C under an atmosphere of 95% air and 5% CO_2. Cells were harvested by trypsinization (1 mg trypsin in 1 ml HBSS containing 5 mM EDTA and 4.2 mM $NaHCO_3$) at room temperature for 2–5 min. Cells were suspended in fresh culture medium and counted with a hemocytometer. After washing, the cells were suspended in Dulbecco's medium without serum at a concentration of $0.5–1.0 \times 10^7$ cells/ml and 100 μl of cell suspension were pipetted into Eppendorf incubation vials. After a preincubation of 1 hr in a shaking water bath 5 μl [6-^{3}H]5FU (1.7 mCi/mmol; final concentration 14.2 μM) were added and the incubation was continued for an additional hour. The reaction was stopped by spinning down the cells in a mini-centrifuge (2 min, 3000 *g*). Blanks were obtained by adding labeled 5FU to cell suspensions just before spinning down; these samples were thereafter treated identically to the other samples. The cell pellets were washed with 120 μl ice-cold saline containing 1 mM 5FU and 1 mM uridine to prevent metabolism of tritiated 5FU. The saline and the medium were combined. The cell pellets were extracted with 100 μl 0.4 M $HClO_4$ for 20 min at 4°C. Denaturated material was spun down with a mini-centrifuge (2 min, 11,500 *g*) and the acid-soluble fraction was neutralized with trioctylamine-Freon [18, 19]. The two layers were separated by centrifugation and the upper-layer was analyzed on PEI-cellulose thin-layer sheets as described in the following section. In subsequent experiments the medium and washing fluid were combined with the extract before analysis. The acid-insoluble pellets were washed several times with 100 μl cold 0.4 M $HClO_4$ until all free tritiated 5FU-metabolites were removed from the pellet (mostly 2–5 times, the last wash containing negligible amounts of radioactivity). The pellet was suspended in 300 μl 0.3 M NaOH and incubated for 3 hr at 37°C to hydrolyze RNA. The unhydrolyzed DNA was precipitated by addition of 50 μl 5 M $HClO_4$. The supernatant, containing the hydrolyzed RNA-nucleotides, was counted after addition of 5 ml Instagel. The pellet was washed 2–5 times with 100 μl 0.4 M $HClO_4$ until the last wash was radioactivity-free. The pellet was solubilized in 200 μl Soluene 350 and radioactivity was estimated after addition of 10 ml Dimilume.

Separation of 5FU-metabolites

The combined washing fluid and medium were analyzed on PEI-cellulose thin layers. Non-radioactive carriers, UTP, UDP, UMP, UDP-glc, 5FU and uridine (5 μl; 1 mM), were first spotted onto the PEI-cellulose sheets to permit localization of the radioactive compounds under u.v. The sheets were developed with distilled water. All nucleotides remained at the origin, while 5FU had an R_f value of 0.67 and FUR of 0.80. These values were identical with those of uracil and uridine. We used uridine to detect the spot of FUR because of the limited availability of this compound. The spots were cut out, and

radioactivity was eluted with 1 ml 0.1 M HCl and 0.2 M KCl for 1 hr and estimated after addition of 10 ml Instagel. The acid-soluble material was analyzed either combined with the washing-fluid and medium or separately. Various systems were used to verify the identity of the metabolites. The migration patterns of 5FU, FUR, FdUMP and FUMP in the various systems were comparable to those of uracil, uridine, dUMP and UMP. Therefore we used UTP, UDP and UDP-glc to locate FUTP, FUDP and FUDP-glc. In system I 10 μl of the extract was spotted onto PEI-cellulose sheets. First development took place in a distilled water–methanol mixture (1:1, v/v) to separate 5FU and FUR from all nucleotides, which remained at the origin. After drying, the sheets were developed in the same direction as far as the 5FU spot in 0.2 M LiCl containing 0.2% formic acid (v/v) at 4°C [20] to separate the nucleotides from each other and from UDP-glc. R_f values are listed in Table 1. The spots were cut out and radioactivity was estimated as described.

System II was used to separate FUMP from FdUMP and from the other metabolites. The PEI-sheets were developed in a mixture of 1 M LiCl in saturated boric acid (0.5 M) and 95% ethanol (1:1, v/v, pH 4.5). R_f values are summarized in Table 1.

Extracts were also analyzed by HPLC using a Partisil-SAX column as described previously [18]. Fractions of 0.3 min were collected and radioactivity was estimated after addition of 5 ml Instagel. The effluent was monitored at 254 and 280 nm to identify the nucleotide pattern of the cells.

RESULTS

Analysis of 5FU-metabolites formed in the absence or the presence of PALA was performed with various methods. Simple separation of 5FU from 5FUR and from the nucleotides could be achieved with PEI-cellulose sheets using water as the eluent. The incubation medium and washing fluid were analyzed with this separation method, since nucleotides formed from 5FU cannot be transported out of the cell and will not be present in the medium. The major part (more than 98%) of the non-metabolized 5FU was present in these fractions. Due to rapid transport of the formed 5FUR out of the cell, the majority (more than 95%) of this nucleoside was also present in the medium. Some unmetabolized 5FU and some 5FUR may leak out of the cells during the washing procedure. Therefore the incubation medium, the washing fluid and the acid-soluble fraction were combined in most experiments to simplify separation procedures.

Table 1. R_f *values of 5FU-metabolites and natural pyrimidines*

Compound	System I	System II
UTP	0.02	0.00
UDP	0.14	0.00
FUMP	0.28	0.08
FdUMP	0.29	0.33
UMP	0.26	0.10
dUMP	0.29	0.41
UDP-glc	0.53	0.17
5FU	0.73	0.69
Uracil	0.73	0.69
FUR	0.79	0.67
Uridine	0.79	0.61

In system I the PEI-cellulose sheets were first developed with methanol–water and then with LiCl–formic acid as described in the text. R_f values were calculated using the front of the methanol–water mixture. System II consisted of the saturated boric acid–LiCl–ethanol mixture.

The LiCl–formic acid system separates all nucleotides from each other, from the base and from the nucleoside. The first eluent (methanol–water) cannot be omitted since single elution with LiCl–formic acid doses not separate UDP-glc and uridine from each other. Furthermore, the methanol–water mixture removes salts, which may interfere with the separation. However, when a mixture of KOH–K_2HPO_4 was used for neutralization the separation of nucleotides was worse than after neutralization with trioctylamine-Freon. Therefore, KOH–K_2HPO_4 neutralization was used only in the initiating experiments.

FUMP and FdUMP could be separated from each other in the boric acid–LiCl system. The sum of radioactivity on these two spots corresponded to the amount found in the LiCl–formic acid system on the monophosphate spot. With both B16 and M5 cells more than 95% of the radioactivity on the monophosphate spot was present as FdUMP. Table 2 shows the data obtained with the LiCl–formic acid system; FdUMP concentrations were corrected for the data obtained with the LiCl–boric acid system. Only a small amount of FUR was formed in B16 cells, while the major part of 5FU-metabolites was present as FUR in IGR3 and M5 cells. More FdUMP was formed in M5 cells than in B16 and IGR3 cells, while the amount of FUTP was higher in B16 cells. In all cell lines only a small amount of 5FU label was present as FUDP or incorporated into DNA. Although the monophosphate was predominantly present as the deoxynucleotide form, the di- and triphosphates appeared to be present as the ribonucleotide. This was determined by periodation of the reaction mixture, which selectively degraded ribonucleotides [21]. After treatment all radioactivity disappeared from the di- and triphosphate spots.

With M5 cells a considerable amount of radioactivity coeluted with UDP-glc. Only small

Table 2. Metabolism of 5FU in intact cells and its modulation by PALA

Cell line	PALA	Metabolite formed FUR	FdUMP	FUTP	FUDP-sugars	RNA	DNA
B16	-	22	4.1	15.6	3.0	8.0	1.6
	+	30	3.8	10.2	3.0	16.0	1.6
IGR3	-	102	5.0	5.4	6.9	1.1	0.15
	+	94	3.8	5.8	13	2.9	0.51
M5	-	194	10.2	7.3	98	6.8	0.54
	+	168	9.1	9.2	81	10.2	0.65

Values (in pmol of metabolite formed/10^6 cells/hr) are means of 2–4 experiments. 10^6 cells were incubated with 14.2 μM 5FU after a pre-incubation of 1 hr. When indicated 1 mM PALA was present during the last 30 min of the pre-incubation. SEM was not greater than 20% of the mean.

amounts of radioactivity on this spot were detectable in extracts of B16 cells. When methanol–water elution was omitted and the PEI-cellulose sheets were only developed with LiCl–formic acid, UDP-glc had the same R_f value as 5FUR. Using this system for extracts of M5 and IGR3 cells, an amount of additional radioactivity appeared on the 5FUR spot, which corresponded to the amount on the UDP-glc spot in system I.

The identity of 5FU-metabolites was confirmed by HPLC analysis by the injection of the reaction mixture on a Partisil SAX column. All radioactivity was recovered from the column. The major part of the radioactivity was present as 5FU and 5FUR (Fig. 1). The elution pattern of the 5FU-metabolites corresponded to that of uridine-metabolites.

The nucleotide profile was recorded simultaneously to check the metabolic condition of the cells. The ATP/ADP ratio was higher than 3, comparable to that measured in cells immediately after harvesting. Concentration of nucleotides did not change during the incubation. The 30-min preincubation with PALA decreased the UTP concentrations by about 50%. The data obtained with HPLC were comparable with the results of PEI-cellulose. In addition to the total amounts of radioactivity, the amount of radioactivity in the separate compounds agreed with the two methods. The pronounced difference between B16 and M5 cells in the synthesis of a 5FU-nucleotide sugar was confirmed with the HPLC analysis.

The effect of PALA on 5FU metabolism was different for the M5, IGR3 and B16 cells (Table 2). PALA hardly affected the incorporation of 5FU in acid-soluble compounds of M5 cells. With B16 cells, PALA reduced the incorporation of 5FU into FUTP. With all cell lines, PALA enhanced the incorporation of 5FU into RNA.

DISCUSSION

The present study demonstrates that metabolism of 5FU is not only limited to the well-known conversion of 5FU to FdUMP and incorporation of 5FU into RNA, but that 5FU-nucleotide sugars can also be formed in considerable amounts. Furthermore, the incorporation into DNA can be demonstrated. The synthesis of all 5FU-metabolites could only be demonstrated using various separation methods. Several methods have been reported for the identification of 5FU-metabolites, including thin-layer chromatography [22, 23], paper chromatography [12] and HPLC [10, 24]. However, all these methods suffer from several disadvantages. Most methods do not separate all 5FU-metabolites from each other. Only with HPLC is a complete separation possible, but this technique is time consuming and laborious when several samples have to be analyzed. With PEI-cellulose sheets [20] various pyrimidine metabolites could be separated with one-directional thin-layer chromatography. The combination of this method with a methanol–water elution also separated UDP-glc from FUR and uridine, providing a complete separation of all 5FU-metabolites except FdUMP from FUMP. However, analysis with the boric acid–ethanol system demonstrated that all 5FU-mononucleotide was present as FdUMP. HPLC elution was used to confirm the results obtained with thin layers.

The pathways leading to the synthesis of 5FU-metabolites are similar to those of the metabolism of the natural pyrimidines uracil, uridine and orotic acid. The pronounced differences between B16, IGR3 and M5 cells partly correlate with differences in enzymes activities. The low amount of FUR formed by B16 cells correlates well with the low activity of uridine phosphorylase in these cells [17]; the higher amount of FUR in IGR3 and M5 cells corresponds to the higher activity of this enzyme in these cells. In B16 cells 5FU is predominantly metabolized by orotate phosphoribosyl transferase, which has considerable activity in the M5 and B16 cell lines [25]. The utilization of this pyrimidine *de novo* enzyme for 5FU phosphorylation in B16 cells probably accounts for the higher amount of 5FU incorporated into the RNA of these cells. The

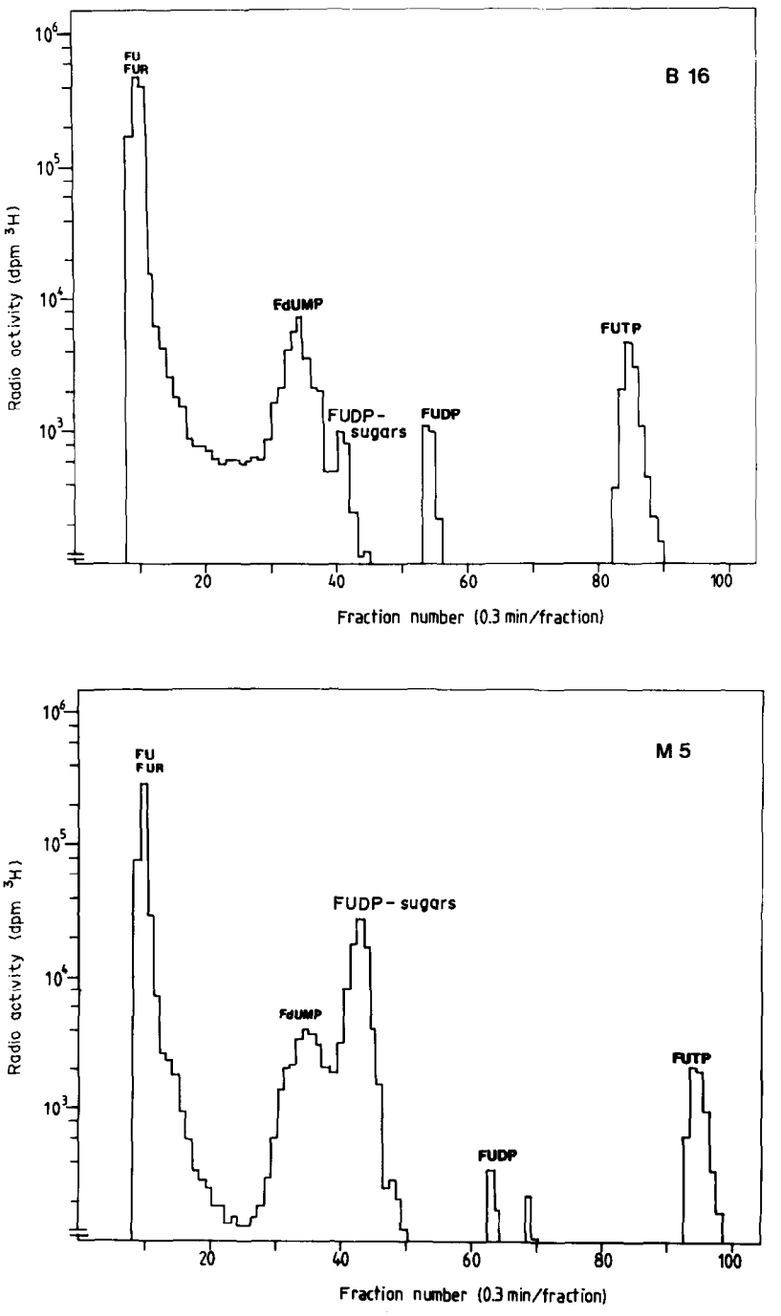

Fig. 1(a,b). Radiochromatograms of B16 and M5 cells incubated with 14.2 μM tritiated 5FU as described in Materials and Methods. A Partisil-SAX column was used at a flow rate of 1.5 ml/min. Elution was started with 100% 0.005 M KH_2PO_4 (pH 4.0) during 5 min, followed by a linear gradient until at 18 min a concentration of 0.25 M KH_2PO_4, 0.50 M KCl (pH 4.5) was reached. The column was eluted for an additional 20 min with this buffer. Fractions of 0.3 min were collected and radioactivity was estimated.

marked stimulation by PALA may be mediated by depletion of pyrimidine nucleotides [15, 16]. A stimulation of 5FU incorporation was also observed in various other cell lines [6, 26]. PALA did not show a significant effect of 5FU incorporation into DNA. Although the DNA fraction had not been purified, the amount of 5FU incorporated appeared to be significant. Comparable incorporation of 5FU into DNA has also been reported by others [5, 6].

M5 cells differed markedly from B16 cells in the high amount of FUDP-sugar that was formed in the former cell line. The difference in initial metabolism of 5FU possibly accounts for the high amount of this nucleotide-sugar. Reyes and Hall [22] earlier suggested a channeling of 5FU into a

different nucleotide pool when 5FU is phosphorylated directly by orotate phosphoribosyl transferase. Also labeled uridine and labeled orotic acid appear in different nucleotide fractions in various organs of the mouse and rat [27, 28]. The FU-nucleotide sugar formed by M5 cells is probably FUDP-glc (or a metabolite of FUDP-glc) since all other nucleotide sugars such as FUDP-galactose are formed from FUDP-glc [29]. Glucosamine and galactosamine, substrates for the direct synthesis of nucleotide-hexosamines, were not present in the medium. With FUR and D-galactosamine the synthesis of various FUDP-hexosamines has been demonstrated [12]. The synthesis of FUDP-hexoses has also been demonstrated by Pogolotti *et al.* [10]. A role of FUDP-sugars in the cytotoxic action of 5FU is not clear. Kessel [30] reported a membrane effect of 5FU and suggested that long-term cytotoxic effects of 5FU could be related to these cell surface effects. A disturbance by 5FU on the synthesis of bacterial cell wall mucocomplex has also been reported [31]. FUDP-sugars could be involved in these processes since UDP-sugars are used for membrane formation. Synthesis of FUDP-sugars could also account for the reported disturbances in glycoprotein synthesis [30]. Although FUDP-sugars may serve as a metabolic source for FUDP, it might also be possible that they contribute to toxic side-effects of 5FU. The present results warrant further studies on the role of FUDP-sugars.

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