Eur J Cancer Clin Oncol, Vol. 23, No. 12, pp. 1869–1881, 1987.
Printed in Great Britain

0277–5379/87$3.00+0.00

Purine Nucleosides as Cell-specific Modulators of 5-Fluorouracil Metabolism and Cytotoxicity

GODEFRIDUS J. PETERS,* EMILE LAURENSSE, ALBERT LEYVA and HERBERT M. PINEDO
Department of Oncology, Free University Hospital, PO Box 7057, 1007 MB Amsterdam, The Netherlands

Abstract—*Purine nucleosides and ribose-5-phosphate (Rib-5-P) were used to modulate the metabolism and cytotoxicity of 5-fluorouracil (5-FU) in order to get a better understanding of the mechanism of action of 5-FU. In extracts from five different cell lines both Rib-5-P and inosine were relatively good precursors for Rib-1-P, but deoxyinosine was a moderate to poor precursor for deoxyRib-1-P. In the human colon carcinoma WiDr and the human epithelial intestinal Intestine 407 inosine enhanced Rib-1-P concentrations 3–6-fold. Incubation with deoxyinosine resulted in the appearance of deoxyRib-1-P in both cell lines in levels comparable to those of Rib-1-P. dIMP had the same effect as deoxyinosine in Intestine 407 cells, but not in WiDr cells. Both inosine and deoxyinosine caused a depletion of phosphoribosyl-pyrophosphate.*

In WiDr cells deoxyinosine (0.1–1.0 mM) clearly potentiated the growth inhibition by 0.1–0.5 μM 5-FU after 24 h of culture, but growth between 24 and 48 h was normal. In Intestine 407 cells the potentiation of 5-FU cytotoxicity by deoxyinosine was even more pronounced at 48 h than at 24 h. In WiDr cells dIMP did not potentiate 5-FU cytotoxicity, but in Intestine 407 cells the effect was comparable to that of deoxyinosine. The lack of potentiation in WiDr was accompanied by a low metabolism of dIMP. Growth inhibition by 5-FU and deoxyinosine could be reversed by thymidine in Intestine 407 cells but not completely in WiDr cells. Since the predominant target of the deoxyinosine–5-FU combination was thymidylate synthase, we analyzed the inhibition of this enzyme by FdUMP and the retention of the inhibition in cell culture. In both cell lines FdUMP was a potent competitive inhibitor of thymidylate synthase with a K_i of between 0.5 and 2 nM. Culture of cells in the presence of 5-FU and deoxyinosine resulted in an almost complete inhibition of thymidylate synthase activity after 24 h but after 48 h the activity was partly recovered. In Intestine 407 cells replenishment of the culture medium at 24 h even enhanced the recovery. Analysis of 5-FU anabolism into nucleic acids demonstrated that deoxyinosine inhibited the incorporation of 5-FU into RNA. It is concluded that in Intestine 407 cells addition of deoxyinosine enhanced the effects of 5-FU on growth inhibition due to increased formation of FdUMP leading to enhanced inhibition of thymidylate synthase. In WiDr cells incorporation of 5-FU into RNA might also contribute significantly to cytotoxicity.

INTRODUCTION

STUDIES on the mechanism of action of 5-FU are hampered by the complex metabolism of this relatively simple antimetabolite [1]. 5-FU can be anabolized directly to FUMP or indirectly via FUR. The contribution of these pathways to the formation of nucleotides depends on the activities of the enzymes catalyzing these reactions and on the availability of the substrates [2]. Measurement of these enzymes can partially predict the route of 5-FU anabolism [3]; although *in vitro* measurement of enzyme activities does not always reflect intracellular metabolism of the substrates. A limited availability of the substrates might influence 5-FU metabolism. In a previous study we measured the concentration of the co-substrate PRPP [4] and found comparable steady state concentrations (between 100 and 200 pmol/10^6 cells) in a panel of

Accepted 7 July 1987.
*To whom correspondence should be directed.
Abbreviations used: 5-FU, 5-fluorouracil; FUR, 5-fluorouridine; FUdR, 5-fluorodeoxyuridine; FUMP, 5-fluoro-uridine-5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; Rib-5-P, ribose-5-phosphate; Rib-1-P, ribose-1-phosphate; dRib-1-P, deoxy-ribose-1-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; OPRT, orotate phosphoribosyl transferase; 5,10-CH_2FH_4, 5,10-methylene tetrahydrofolate; TCA, trichloroacetic acid; IC_{50}, concentration that causes 50% growth inhibition.
Portions of this work have been presented at the 5th International Symposium on Human Purine and Pyrimidine Metabolism, San Diego, CA, U.S.A., 1985.

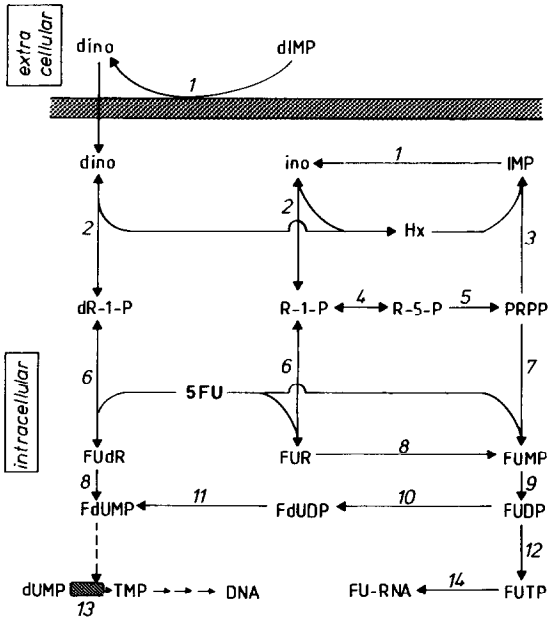

Fig. 1. Schematic outline of intra- and extracellular metabolism of (deoxy)inosine and dIMP in relation to 5-FU. The enzymes catalyzing these reactions are; 1, phosphatases and 5'-nucleotidases; 2, purine nucleoside phosphorylase; 3, hypoxanthine phosphoribosyl transferase; 4, phosphoribomutase; 5, PRPP synthetase; 6, pyrimidine nucleoside phosphorylase; 7, orotate phosphoribosyl transferase; 8, pyrimidine nucleoside kinases; 9, nucleoside monophosphate kinase; 10, ribonucleotide reductase; 11, 5'-nucleotidase; 12, nucleoside diphosphate kinase; 13, thymidylate synthase; 14, RNA polymerase.

six cell lines of different histological origin. Consumption of PRPP and enzyme activities correlated quite well with intracellular 5-FU metabolism [5].

Although 5-FU cytotoxicity can be related to some extent with the activities of enzymes catalyzing its metabolism [6, 7], the actual determinants of cell growth inhibition are the incorporation of 5-FU into RNA or the binding of FdUMP to thymidylate synthase leading to inhibition of DNA synthesis. It has been postulated that 5-FU incorporation into DNA may also contribute to 5-FU cytotoxicity. The mechanism of cytotoxicity might vary in each different cell line or tissue. Biochemical modulation of the activation pathways of 5-FU offers the possibility to a selective enhancement of e.g. FdUMP formation and to determine whether FdUMP inhibition of thymidylate synthase is an important mechanism of action in a specific cell line.

Synthesis of FdUMP or FUMP can be selectively enhanced by providing a higher concentration of one of the co-substrates, dRib-1-P or Rib-1-P, respectively. Since these pentose phosphates cannot pass the cellular membrane, precursors such as purine nucleosides (Fig. 1) can be used. These nucleosides can easily be transported into the cell by facilitated diffusion [8]; their phosphorolysis is catalyzed by purine nucleoside phosphorylase yielding hypoxanthine and pentose phosphates, which can serve as co-substrates for 5-FU metabolism. The possibility of using purine nucleosides as precursors for 5-FU anabolism has already been recognized for several decades [9, 10]. In a variety of systems either inosine or deoxyinosine have shown to be able to increase the growth inhibition by 5-FU [11–14] and to enhance the antitumor activity [15]. It has also been shown that deoxyinosine or inosine can protect cells against 5-FU [11, 16]. Also purine nucleotides can serve as precursors for pentose phosphates [17]. It has also been demonstrated that nucleosides might be useful to study the mechanism of action, since a selective stimulation of a certain pathway is obtained [18].

All purine nucleosides affect 5-FU metabolism differently. Inosine affects the cytotoxicity of 5-FU by enhancing incorporation into RNA [13], while deoxyinosine can enhance the FdUMP pool [12, 18] leading to increased inhibition of DNA synthesis. However, both inosine and deoxyinosine can decrease 5-FU incorporation into RNA [11], thus preventing a lack of potentiation. Thus, a detailed study of the effects of inosine and deoxyinosine on 5-FU cytotoxicity and metabolism might lead to a better understanding of the mechanism of action of 5-FU in specific cell lines. In a panel of cell lines, which were used for extensive studies of 5-FU metabolism [3], we determined the capacity of purine nucleosides to replace the pentose phosphates as co-substrates for 5-FU anabolism. We also included Rib-5-P in these studies since the concentration of this pentose phosphate is usually higher in cells as the equilibrium of the phosphoribomutase reaction is at the Rib-5-P site [19]. Furthermore Rib-5-P is a precursor for both Rib-1-P and PRPP. In two selected cell lines, both of human intestinal origin, we studied the synergism of 5-FU and deoxyinosine, and the effect of these nucleosides on pentose phosphate concentration and PRPP. These results are related with the effect of deoxyinosine on 5-FU incorporation into nucleic acids and on FdUMP binding and on the retention of FdUMP binding to thymidylate synthase.

MATERIALS AND METHODS

Materials

The origin of the various cell lines, culture media and fetal bovine serum have been described previously [3]. 5-FU, FUR, FUdR, FdUMP, dUMP, hypoxanthine, inosine, deoxyinosine, Rib-1-P, dRib-1-P and dIMP were obtained from Sigma, St. Louis, MO, U.S.A., while PRPP, RNase, purine nucleoside phosphorylase and orotate phosphoribosyltransferase were from Bochringer, Mannheim, F.R.G. The radiochemicals [6-^{14}C]5-FU, [6^{3}H]5-FU, [5-^{3}H]dUMP, [carboxyl-^{14}C]orotic acid and [8-^{14}C]hypoxanthine were obtained from New England Nuclear Corporation, Dreieichenhain,

F.R.G. Prepacked HPLC columns LiChrosorb-10RP18 (length 25 cm, ID 4.6 mm, particle size 10 μm) and Partisil-SAX (length 25 cm, ID 4.6 mm, particle size 10 μm) were obtained from Chrompack, Middelburg, The Netherlands. All other chemicals were of analytical grade quality.

Cell culture

Cells were routinely cultured in 10% undialyzed, heat-inactivated fetal bovine serum in 20 mM hepes-buffered Dulbecco's MEM medium in 75 cm^2 culture flasks at 37°C under an atmosphere of 5% CO_2. Growth inhibition experiments were performed with 15% dialyzed fetal bovine serum in 6-well (10 cm^2 per well) cluster plates essentially as described previously [3]. Cells were continuously exposed to drugs, nucleosides and dIMP; the extent of growth inhibition was calculated as described previously [3]. The media in which the cells had been cultured were collected after 24 and 48 h; deproteinized with trichloroacetic acid (final concentration 5%; w/v), neutralized with alamine–Freon [20] and frozen until analyses could be performed.

Enzyme assays

Enzyme activities were measured in extracts prepared from freshly isolated cells or from cell pellets frozen at −70°C, as described previously [3]. Shortly, the rate of 5-FU metabolism was measured at a substrate concentration of 0.27 mM [6-^{14}C]5-FU in the presence of 5 mM $MgCl_2$. Co-substrates (Rib-1-P or dRib-1-P) and precursors for these co-substrates (Rib-5-P, inosine or deoxyinosine) were present at a final concentration of 2.5 mM. The activity of thymidylate synthase was assayed by following the release of tritium from [5-^{3}H]dUMP [21] as described previously [3].

Analysis of metabolites in culture medium

The concentrations of nucleosides and bases were determined with HPLC on a reversed-phase column (LiChrosorb 10-RP-18) using isocratic elution (1.5 ml/min; 50 mM KH_2PO_4, pH 4.5 containing 6% methanol). Hypoxanthine, inosine and deoxyinosine were eluted afer 5.0, 6.4 and 8.4 min, respectively. The concentration of dIMP was determined with HPLC using an anion exchange column (Partisil-SAX) using isocratic conditions (1.5 ml/min; 0.13 M KH_2PO_4, 0.25 M KCl, pH 4.4). Deoxyinosine, hypoxanthine and dIMP were eluted after 4.2, 4.4 and 5.3 min, respectively.

Assay of PRPP, Rib-1-P and dRib-1-P

The concentration of PRPP was measured using the method based on $^{14}CO_2$ release from [carboxyl-^{14}C]orotic acid [22, 23]. The effect of nucleosides on PRPP concentration was determined as described previously [4, 24].

The concentration of Rib-1-P was determined with a method slightly modified from those described previously [25, 26]. Briefly, a cell pellet (1–2 × 10^6 cells) was suspended in 60 μl Tris–HCl buffer (50 mM; pH 7.4) containing 1 mM EDTA. The suspension was heated during 5 min at 95°C in an Eppendorf incubator in order to denature interfering proteins. Thereafter the tubes were chilled in ice. The denatured protein was spun down. For the Rib-1-P assay 10 μl purine nucleoside phosphorylase (3 μg) and 10 μl [8-^{14}C]hypoxanthine (6.2 nmol, 18 mCi/mmol) were added to the supernatant. The tubes were incubated for 30 min in a shaking waterbath at 37°C. The reaction was terminated by heating at 95°C for 3 min followed by addition of 20 μl 5 mM hypoxanthine and inosine. Denatured protein was spun down and the supernatant was analyzed on thin-layer sheets as described previously for the purine nucleoside phosphorylase assay [27]. Inosine and deoxyinosine have the same R_f value. The calibration curve included Rib-1-P concentrations ranging from 1 to 50 μM. In order to check complete conversion of Rib-1-P and its recovery, a known amount of Rib-1-P was added to the cell pellet just before or just after the first heating step.

The effect of nucleosides and dIMP on the concentrations of Rib-1-P and dRib-1-P was determined using an incubation procedure similar to that used for PRPP. Briefly, cells were harvested, suspended in Dulbecco's medium without serum, incubated for 1 h at 37°C in a shaking waterbath and nucleosides were added. Incubations were proceeded for an additional 1 or 2 h and were stopped by spinning down the cells in a minicentrifuge. The medium was pipetted off and the cell pellets were immediately frozen at −70°C. Freezing did not affect the Rib-1-P or dRib-1-P concentrations.

5-FU incorporation into RNA, DNA and binding of FdUMP to thymidylate synthase

Measurement of 5-FU incorporation into RNA was performed after incubation with [6-^{3}H]5-FU, according to methods slightly modified from those described previously [3]. Cells were incubated in 96-well Millititer GV plates which were coated at the bottom with hydrophilic Durapore membrane with a 0.22 μm pore size (Millipore Corporation, Bedford, Massachusetts, U.S.A.). Each well contained 10^5–10^6 cells in 100 μl hepes-buffered Dulbecco's MEM medium without serum. After a preincubation at 37°C under 5% CO_2 10 μl nucleosides were added to the wells followed after 30 min by 10 μl [6-^{3}H]5-FU (1.7 or 3.4 nmol; 685 mCi/mmol, respectively). After an incubation of 0–4 h, the whole plate was placed on a special

Table 1. Activity of pyrimidine nucleoside phosphorylase in the presence of inosine, deoxyinosine or Rib-5-P

Cell line	Precursor		
	Rib-5-P	Inosine	Deoxyinosine
B16	0.5 ± 0.1	n.d.	n.d.
IGR3	11.3 ± 0.1	6.9 ± 0.2	2.5 ± 0.2
M5	2.9 ± 0.8	5.8 ± 1.0	1.4 ± 0.2
WiDr	0.5 ± 0.2	0.6 ± 0.1	0.1 ± 0.05
Intestine 407	1.2 ± 0.2	0.9 ± 0.1	0.3 ± 0.13
H35	2.4 ± 0.1	4.2 ± 0.1	1.9 ± 0.1

Activities (means ± S.E.) are given in nmol/h per 10^6 cells and are from 3–5 separate experiments. n.d., not done.

Millititer vacuum holder (Millipore) and the medium was filtered through the filter. Subsequently the cells were precipitated on the filter with 150 μl ice-cold 5% TCA. After filtration of the acid-soluble fraction through the filter this procedure was repeated three times, followed by four washes with 100 μl H_2O and four washes with 100 μl 70% ethanol. After drying the filter could be removed from the 96-well plate with a Millititer filter punch (Millipore). Radioactivity was determined by addition of 100 μl Soluene-350 to the filters followed after 10 min by 5 ml Dimilume. The values obtained by this procedure represent the incorporation of 5-FU into RNA and DNA and the binding of 5-FdUMP to thymidylate synthase. In order to separate RNA from other fractions the washing steps with 70% ethanol were omitted and replaced by four additional washes with 100 ml H_2O. Thereafter the acid-insoluble fractions on these filters were incubated for 20 min with RNase (15 μg in 100 μl Tris–HCl, containing 1 mM EDTA at pH 7.4) at 37°C. This reaction was stopped as described above by filtration of the fluid followed by four washes with ice-cold TCA, four washes with water and four washes with 70% ethanol. Thereafter the radioactivity on the filters could be determined. This radioactivity will represent the amount of 5-FU incorporated into DNA and the amount of 5-FU bound to thymidylate synthase in the form of a FdUMP–thymidylate synthase–folate complex.

RESULTS

Enzyme activities

A panel of six cell lines from different histological origins was assayed for their capacity to use purine nucleosides as precursors for pentose phosphates. This was performed by measuring the conversion of 5-FU to FUR or to FUdR in the presence of inosine or deoxyinosine (Table 1). Since pentose phosphates are usually present in the cell as Rib-5-P [19, 28] we also used this pentose phosphate as a substrate. The highest absolute activity with Rib-5-P was found in IGR3 cells and the lowest activity in WiDr and B16 cells.

With inosine as a precursor the highest absolute activity was also found in IGR3 cells, but activity in the M5 cells was comparable. Lowest activity was observed in the human intestine cell lines WiDr and Intestine 407. With deoxyinosine as a precursor the absolute activities were much lower than with inosine. This is in contrast to the activities with Rib-1-P and dRib-1-P, which were comparable in all these cell lines [3].

The absolute values were compared with data obtained with the natural co-substrates Rib-1-P and dRib-1-P, which were reported previously [3]. The percentages given in Table 2 were obtained from experiments in which the activity of pyrimidine nucleoside phosphorylase was measured both with the natural co-substrate and with the precursor.

With Rib-5-P as precursor the highest relative activity was found in B16 cells and the lowest in WiDr cells. With both inosine and deoxyinosine the lowest relative rate was observed in IGR3 and WiDr cells, while the highest rate was observed in Intestine 407 cells.

Concentrations of Rib-1-P, dRib-1-P and PRPP

The Rib-1-P assay had a detection limit of 100 pmol Rib-1-P per assay and was linear up to at least 5 nmol per incubation mixture (Fig. 2), both with standards and with standards added to cell extracts. The recovery of Rib-1-P which was added before the first heating step was > 95%. After 30 min conversion of Rib-1-P to inosine was complete; a 60 min incubation gave similar results. It was assumed that the amount of dRib-1-P in cells was negligible [29], and that [^{14}C]hypoxanthine was only converted to [^{14}C]inosine with the physiological intracellular Rib-1-P as co-substrate. This assumption was verified by measurement of the Rib-1-P concentration with HPLC; the cell extract was incubated with purine nucleoside phosphoryl-

Table 2. Relative rate of pyrimidine nucleoside phosphorylase in the presence of precursors

Cell line	Precursor		
	Rib-5-P	Inosine	Deoxyinosine
B16	94 ± 16	n.d.	n.d.
IGR3	40 ± 5	24 ± 5	8 ± 1
M5	34 ± 7	50 ± 7	11 ± 2
WiDr	30 ± 6	33 ± 5	7 ± 1
Intestine 407	70 ± 6	86 ± 8	19 ± 5
H 35	31 ± 4	47 ± 4	13 ± 3

Values (means ± S.E.) are percentages of the enzyme activities with Rib-1-P (for Rib-5-P and inosine) or with dRib-1-P (for deoxyinosine) as co-substrates performed in the same experiment. Values are from 3–4 separate experiments. n.d., not done.

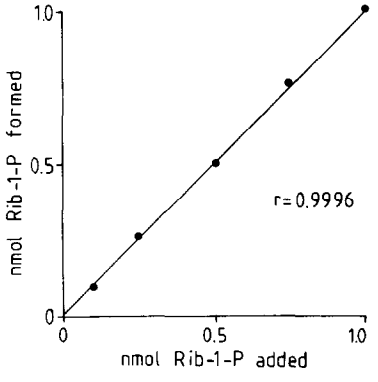

Fig. 2. Linearity of the Rib-1-P assay.

ase and 50 μM non-radiolabeled hypoxanthine. The formed inosine was determined with HPLC. No deoxyinosine could be detected.

Intracellular inosine or deoxyinosine might affect the Rib-1-P assay. In cell suspensions incubated with 1 mM inosine, the major part of the nucleoside would be in the medium and would be lost after centrifugation of the cells. Inosine in the cells might be higher than physiological levels but was diluted by the Tris–EDTA buffer to a very low concentration. We added excess (2.5 nmol) non-radiolabeled inosine to the Rib-1-P assay mixture. The conversion of [^{14}C]hypoxanthine to [^{14}C]inosine was not altered. This means that Rib-1-P measurements in cells incubated with inosine were accurate.

The concentration of Rib-1-P in cells was measured immediately after harvesting, and after 1 and 2 h. The concentrations of Rib-1-P were between 0.2 and 1.5 pmol/10^6 cells with all cell lines tested (data for B16, M5 and IGR3 are not shown; data for WiDr and Intestine 407 in Table 3). After incubation the Rib-1-P concentrations were comparable to those before incubation, in contrast to PRPP concentrations [4, 24]. Incubation with 1 mM inosine increased the Rb-1-P concentration 3–6-fold. Incubation of cells with deoxyinosine resulted in the appearance of dRib-1-P. The assumption was made that the Rib-1-P pool will not be altered by incubation with deoxyinosine and that the additional radioactivity at the inosine spot was [^{14}C]deoxyinosine, and represented dRib-1-P present in the cells [29]. The incubation with dIMP also enhanced dRib-1-P in both cell lines, but to a lower extent in WiDr cells.

In all cell lines tested inosine decreased the concentration of PRPP down to about 20% of the original concentration (Table 4). Deoxyinosine also decreased the concentration of PRPP in all cell lines, but in IGR3 cells this decrease was only down to about 50% of the original concentration. The effect of dIMP on PRPP concentration was only studied in WiDr and Intestine 407 cells. PRPP concentration was decreased in both cell lines after incubation with dIMP.

Effect of deoxyinosine and inosine on growth inhibition by 5-FU

For several reasons the WiDr and Intestine 407 cell lines were selected for growth inhibition studies in combination with deoxyinosine and inosine. Firstly, these cell lines showed the lowest and highest relative rate of usage of purine nucleoside precursors. Secondly, both cell lines are from human intestinal origin. Thirdly, WiDr has a very low activity of thymidylate synthase, while Intestine 407 has a relatively high activity [3]. Furthermore, both cell lines have a comparable activity of OPRT which catalyzes the direct activation pathway for 5-FU [3, 4], utilizing PRPP, so interference of this pathway will be comparable.

Inosine did not inhibit cell growth nor potentiate the growth inhibition by 5-FU on WiDr and Intestine 407 cells (data not shown). Deoxyinosine inhibited growth of WiDr cells only at the highest concentration tested (1 mM). Deoxyinosine was tested with 5-FU concentrations which did not or only slightly affect the growth inhibition. The IC_{50} values for Intestine 407 and WiDr were about 1.7 and 0.7 μM [3]. Therefore we chose 0.1 and

Table 3. Effect of inosine and of deoxyinosine and dIMP on the intracellular concentrations of Rib-1-P and dRib-1-P

Addition	Time (h)	Compound	WiDr	Intestine 407
None	0	Rib-1-P	0.26 ± 0.04 (6)	1.10 ± 0.18 (5)
None	1	Rib-1-P	0.38 ± 0.05 (6)	0.79 ± 0.14 (5)
None	2	Rib-1-P	0.39 ± 0.07 (6)	0.72 ± 0.10 (5)
Inosine	2	Rib-1-P	1.94 ± 0.44 (4)	2.54 ± 0.19 (3)
Deoxyinosine	2	dRib-1-P	1.56 ± 0.29 (5)	2.25 ± 0.44 (3)
dIMP	2	dRib-1-P	0.27 ± 0.06 (3)	2.05 ± 0.63 (3)

Values (in pmol/10^6 cells) are means ± S.E. for the number of experiments indicated within parentheses. Cells were incubated for the time periods indicated. These values are corrected for the Rib-1-P concentrations, which were present after 2 h in controls without additions. Inosine, deoxyinosine and dIMP were present in the incubation mixture from 1 to 2 h.

Table 4. Effect of inosine, deoxyinosine and dIMP on the PRPP concentration

Cell line	Initial PRPP concentration	Relative concentration (%)		
		Inosine	Deoxyinosine	dIMP
B16	200 ± 20	18.1 ± 4.7	13.4 ± 3.7	n.d.
IGR3	87 ± 10	21.8 ± 5.1	51.6 ± 10.8	n.d.
M5	111 ± 19	12.9 ± 5.4	22.7 ± 2.6	n.d.
WiDr	131 ± 19	18.1 ± 2.1	23.9 ± 3.4	22.1 ± 2.9
Intestine 407	84 ± 22	30.2 ± 9.5	11.9 ± 4.1	22.5 ± 6.6

Values are means ± S.E. of 3–5 separate experiments. Deoxyinosine, inosine and dIMP were present for 2 h at a concentration of 1 mM final concentration and were added 1 h after suspension of the cells. The PRPP concentration at this time point is given in pmol/10^6 cells. n.d., not done. The PRPP concentration at the end of the incubation was set at 100%. The effects of 0.4 mM inosine and deoxyinosine were comparable to those of 1 mM.

0.5 μM 5-FU for the WiDr cells and 0.5 and 1 μM for the Intestine 407 cells. Deoxyinosine clearly potentiated the effect of 5-FU in both cell lines (Fig. 3). However, there was a distinct difference between Intestine 407 and WiDr. At 0.1 μM 5-FU the potentiation in WiDr cells was only observed at 1.0 mM deoxyinosine after 24 h, but after 48 h this effect was less. At 0.5 μM 5-FU cell kill was observed after 24 h in combination with 0.4 and 1.0 mM deoxyinosine, but at 48 h relative growth inhibition was less than at 24 h. In Intestine 407 cells the inhibition by 0.5 μM 5-FU and deoxyinosine was comparable at 24 and 48 h; but at 1 μM 5-FU the potentiating effect was even more pronounced at 48 h.

Effect of dIMP on growth inhibition by 5-FU

In order to get a more selective modulation of growth inhibition, we used dIMP as an indirect source for dRib-1-P. The growth inhibition by 5-FU in WiDr cells was not affected by dIMP (Fig. 4B). However, in Intestine 407 the growth inhibition by 5-FU was clearly enhanced by dIMP (Fig. 4A) at the highest concentration of dIMP even leading to cell kill. The effect was comparable to that of deoxyinosine.

Analysis of culture media

In the culture media of cells treated with 5-FU and deoxyinosine or dIMP we measured the concentrations of deoxyinosine and dIMP and of their metabolites (Table 5). Striking differences were observed, which were partly in accordance with the differences in influence on growth inhibition. In both cell lines deoxyinosine was rapidly broken down to hypoxanthine. However, in WiDr at an initial concentration of 1 mM deoxyinosine an equilibrium was reached after 24 h since the amount of hypoxanthine did not increase further. In Intestine 407 cells degradation of deoxyinosine continued after 24 h. In both cell lines inosine was found in the culture medium which might be formed either directly from hypoxanthine in a reaction catalyzed by purine nucleoside phosphorylase or from dephosphorylation of IMP, which is a product of phosphoribosylation of hypoxanthine (Fig. 1).

In both cell lines dIMP was dephosphorylated by an extracellular enzyme, either an ecto-5′-nucleo-

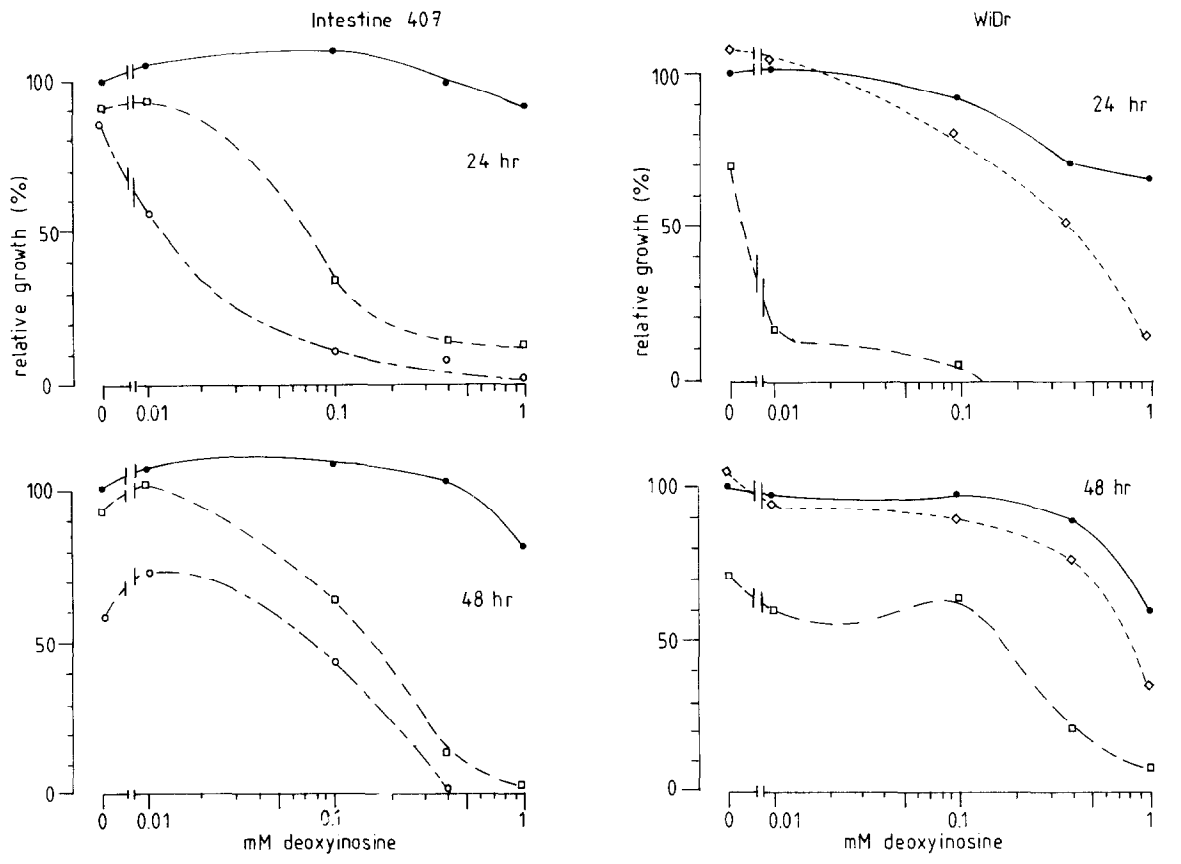

Fig. 3. Growth inhibition by 5-FU in combination with deoxyinosine in Intestine 407 and WiDr cells. Values are means of 3–5 separate experiments. S.D. was less than 10%. 5-FU was present at 0.1 μM (◊--◊), 0.5 μM (□--□) and 1 μM (○--○) or not present (●—●).

tidase or an ecto-phosphatase. In Intestine 407 cells the formation of deoxyinosine and hypoxanthine continued after 24 h; after 48 h the majority of dIMP was broken down to hypoxanthine. However, in WiDr cells dephosphorylation of dIMP did not continue after 24 h. The major part of dIMP was still present after 48 h.

Effect of thymidine on growth inhibition by 5-FU and deoxyinosine

In order to determine whether growth inhibition by 5-FU and deoxyinosine or dIMP was mediated by inhibition of DNA synthesis, we added thymidine to the cultures. Thymidine will bypass the inhibition of thymidylate synthase. In both cell lines thymidine reversed the growth inhibition by 5-FU and deoxyinosine. This effect was most pronounced in Intestine 407 cells (Fig. 5A). In these cells both 2 and 10 μM thymidine almost completely reversed growth inhibition induced by 5-FU and deoxyinosine or dIMP. The growth rate between 24 and 48 h was comparable to that of control cultures. However, in WiDr cells the kinetics of growth inhibition were different. Addition of 10 μM thymidine even enhanced the growth inhibition caused by 5-FU and deoxyinosine or dIMP (Fig. 5B). This effect was more pronounced with dIMP. After 24 h the growth rate in the presence of thymidine was comparable to that of control cells.

The effect of thymidine was also studied on growth inhibition by 5-FU alone (at concentrations shown in Fig. 5). In Intestine 407 cells, 2 and 10 μM thymidine completely reversed growth inhibition after 24 and 48 h; in WiDr the reversal was intermediate at both time points (data not shown).

Inhibition of thymidylate synthase

In both cell lines we analyzed the inhibition of thymidylate synthase by FdUMP. Activity of thymidylate synthase, assayed at an optimal concentration of 5,10-CH_2FH_4, was four times higher in Intestine 407. In both cell lines the K_m for dUMP was comparable. The mode of inhibition by FdUMP was competitive (Fig. 6).

Not only the extent of enzyme inhibition but also the retention of enzyme inhibition is important to prolong the growth inhibition. Therefore cells were incubated with 5-FU and deoxyinosine to enhance FdUMP formation and enzyme activity was measured in cell extracts after 24 and 48 h at an optimal and a suboptimal dUMP concentration. Dilution of cell extracts with assay buffer will decrease endogen-

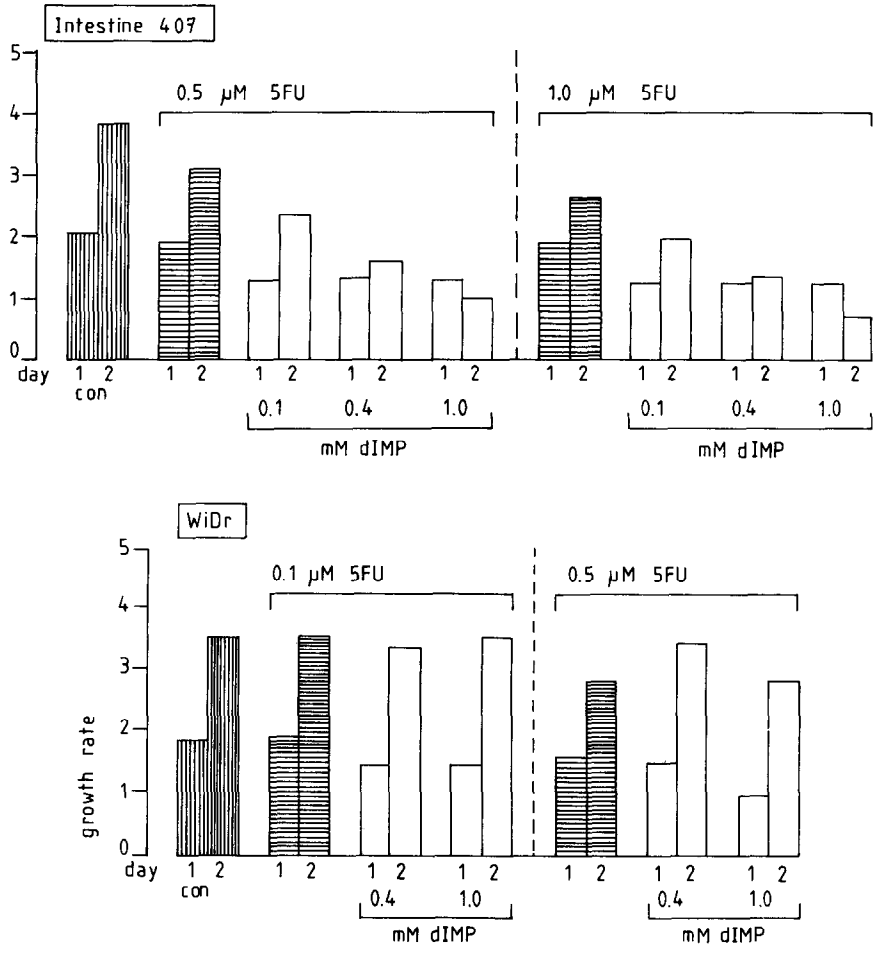

Fig. 4. Growth inhibition by 5-FU in combination with dIMP in Intestine 407 cells and WiDr cells. Bars represent means of 3–4 separate experiments. S.D. was less than 10%. Con, control cultures without additions.

Table 5. Concentrations of dIMP, deoxyinosine, hypoxanthine and inosine in medium of WiDr and Intestine 407 cells

Treatment	Time (h)	WiDr				Intestine 407			
		dIMP	Deoxyinosine	Hypoxanthine	Inosine	dIMP	Deoxyinosine	Hypoxanthine	Inosine
0.4 mM dIMP	24	294	59	65	—	155	95	97	—
	48	188	84	132	—	40	118	222	—
1.0 mM dIMP	24	683	69	79	—	647	179	133	—
	48	642	117	136	—	222	293	305	—
0.4 mM deoxyinosine	24	—	275	120	2.8	—	196	190	14
	48	—	275	170	2.7	—	115	264	18
1.0 mM deoxyinosine	24	—	750	243	6	—	727	249	22
	48	—	700	284	14	—	542	415	34

Values (in μM) are means of 2–3 separate experiments.

ous levels of both FdUMP and dUMP. So, the observed inhibition of thymidylate synthase will only be the result of FdUMP bound to thymidylate synthase in the ternary complex. In both WiDr and Intestine 407 cells thymidylate synthase was almost completely inhibited when assayed at a low concentration of dUMP. At an optimal dUMP concentration inhibition was less in WiDr cells. However, the most pronounced difference between both cell lines was the retention of inhibition. When the culture medium was not refreshed at 24 h the activity of thymidylate synthase in WiDr cells was partly recovered after 48 h, but to a lower extent in Intestine 407. However, this difference between both cell lines was more pronounced when the medium of the cells was refreshed after 24 h. The

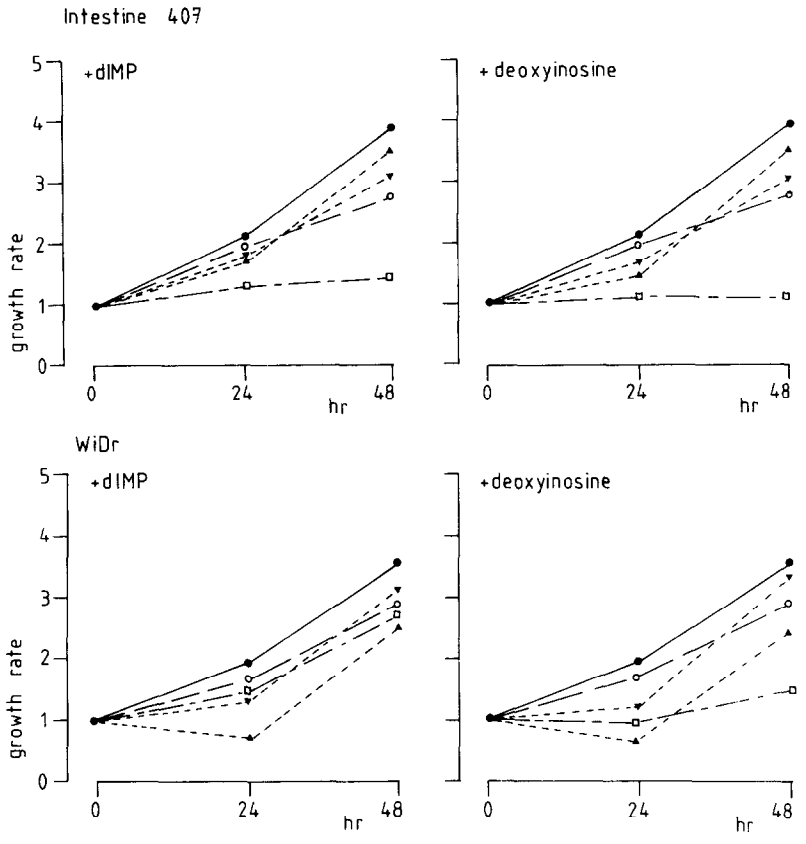

Fig. 5. Prevention by thymidine of growth inhibition by 5-FU with deoxyinosine or dIMP. Figures represent one experiment out of three. Solid lines are those from cultures without any addition. ○--○, *5-FU alone;* □--□, *5-FU with deoxyinosine and/or dIMP;* ▼--▼, *5-FU and deoxyinosine or dIMP, with* 2 μM *thymidine;* ▲--▲, *5-FU, deoxyinosine or dIMP with* 10 μM *thymidine. Deoxyinosine and dIMP were added at* 1 mM, *while 5-FU was added at* 0.5 μM *in WiDr cells and* 1.0 mM *in Intestine* 407 *cells.*

recovery of thymidylate synthase was not enhanced in WiDr. However, in Intestine 407 cells the activity of thymidylate synthase was almost normal, at least at optimal dUMP concentrations, at suboptimal dUMP concentration recovery was about 50%.

Effect of deoxyinosine on 5-FU metabolism

In order to determine whether deoxyinosine affected 5-FU incorporation into RNA we measured 5-FU incorporation into RNA (Table 6). The methods described in this paper yielded similar results as a more laborious conventional assay described previously [3]. The washing steps can be performed more conveniently and thoroughly using the 96-well Millititer plates coated with Durapore filter. Furthermore, more samples can be analyzed in the same time period, since up to 96 wells can be processed in the same time period. Alkaline digestion of RNA was substituted by RNase treatment. Results were similar to those described previously [3]. The incorporation of 5-FU into RNA was linear with the 5-FU concentration (Table 6). Incorporation was also linear with time (data not shown). This was found with both cell lines.

Deoxyinosine inhibited 5-FU incorporation into RNA in both cell lines. Incorporation was reduced down to 36 ± 5 and 38 ± 1% (means ± S.E.) of control levels of WiDr and Intestine 107 cells, respectively. Incorporation of 5-FU into RNase stable high-molecular weight material was also determined. This high-molecular weight material consists of 5-FU incorporation into DNA and the FdUMP binding to thymidylate synthase [30, 31]. Since incorporation of 5-FU into DNA is usually very low [32] and since the FdUMP thymidylate synthase complex is stable to TCA precipitation [33], this RNase stable material will be predominantly FdUMP bound to thymidylate synthase. A slight increase of radioactivity in this fraction was observed in WiDr cells at 0.4 mM deoxyinosine, but not Intestine 407 cells.

DISCUSSION

In this study we demonstrate that deoxyinosine can enhance growth inhibition by 5-FU, but that the extent and time course of potentiation were different for the two human colon cell lines which were used. Modulation with deoxyinosine was used

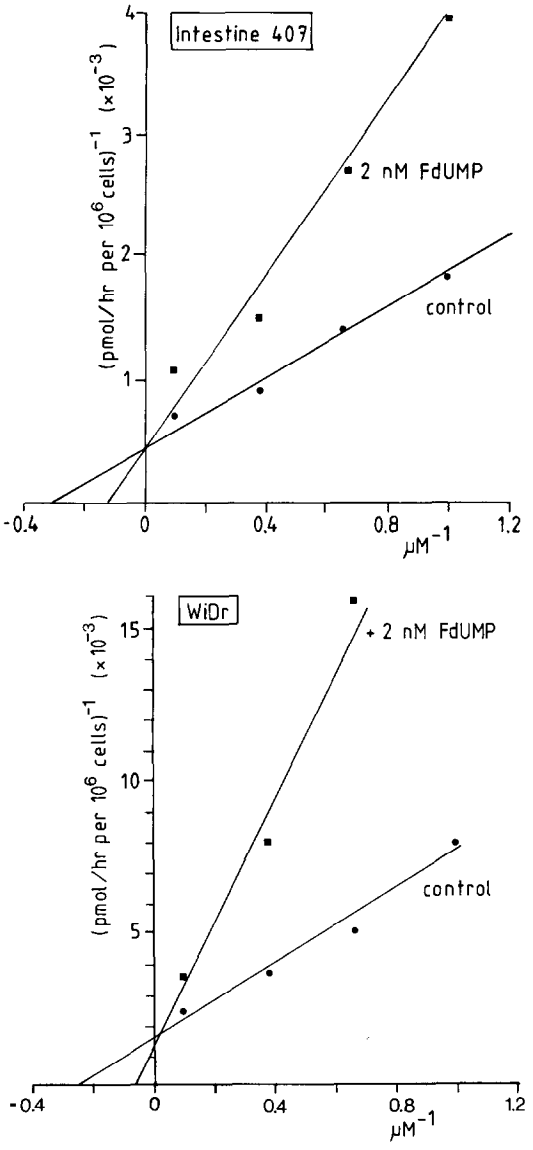

Fig. 6. Lineweaver-Burk plots of thymidylate synthase in Intestine 407 cells and WiDr cells. The K_m values for dUMP were 2.78 ± 0.34 and 3.72 ± 0.28 μM for Intestine 407 and WiDr cells, respectively, and the K_i values for FdUMP 1.84 ± 0.35 and 0.50 ± 0.14 nM respectively. V_{max} values were 1361 ± 57 and 379 ± 41 pmol/h per 10^6 cells, respectively. Values are means ± S.E. of three separate experiments. Enzyme inhibition by FdUMP was competitive in respect to dUMP. The plots are of one representative experiment.

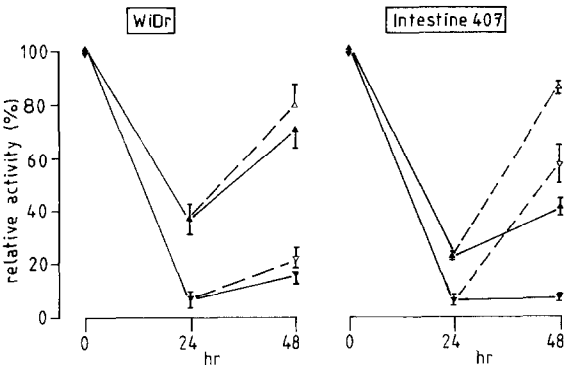

Fig. 7. Recovery of thymidylate synthase in Intestine 407 and WiDr cells. Solid lines (connecting closed symbols) represent the activity in the continuous presence of 5-FU and deoxyinosine (0.4 mM). 5-FU concentrations were 1 and 0.5 μM for Intestine 407 and WiDr, respectively. Broken lines (connecting open symbols) represent culture in drug-free medium, after replenishment at 24 h. Values are means ± S.E. of three separate experiments. ▼, ▽; activity of thymidylate synthase assayed at 1 μM dUMP; ▲, △; activity assayed at 10 μM dUMP.

for further characterization of the mechanism of action of 5-FU. Potentiation of 5-FU by deoxyinosine has already been shown by others in different systems [9, 12, 15, 18, 31]. However, it has also been shown that purines protected cells from 5-FU toxicity [16, 34]. This differential effect of purines on 5-FU cytotoxiciy was used to study the mechanism of action of 5-FU. It has been reported that a protection by purines of 5-FU cytotoxicity was accompanied by a decrease in PRPP [16, 31]. Since PRPP is an essential co-substrate for the direct conversion of 5-FU to FUMP, a depletion of PRPP will decrease the rate of this reaction. In cells in which this pathway is the only one responsible for 5-FU anabolism, purines will thus protect these cells from the cytotoxic effect of 5-FU. Both WiDr and Intestine 407 cells were not protected (by either inosine or deoxyinosine) leading to the conclusion that the direct conversion of 5-FU to FUMP is not the only pathway leading to activation of 5-FU. However, this does not imply that under physiological conditions (without inosine) the activation pathway via FUR to FUMP is more important, since the increase in Rib-1-P concentrations (induced by inosine) is not accompanied by an enhanced growth inhibition. It might even be possible that a lower rate of the direct conversion of 5-FU to FUMP in the presence of inosine was compensated by a higher rate of the indirect pathways.

The increase in Rib-1-P concentrations induced by inosine probably represent steady state concentrations, which will be maintained longer. Measurement of Rib-1-P concentrations after a longer time period yielded the same levels, as was also found by others [35]. The same holds for dRib-1-P. The degradation of deoxyinosine to hypoxanthine continued after 24 h, thus providing a continuous supply for dRib-1-P, which will enhance the FdUMP formation [12]. Probably, under these conditions this will be the only pathway for 5-FU anabolism, since PRPP is also depleted. The same mechanism is responsible for the potentiation of 5-FU by dIMP. This nucleotide, which has a better solubility than deoxyinosine, enhanced the dRib-1-P concentrations in Intestine 407 cells and decreased PRPP concentrations. The lack of potentiation of 5-FU in WiDr correlates with the low ecto-phosphatase and ecto-nucleotidase activity in these cells compared to Intestine 407 cells [3]; this is also reflected by

Table 6. Effect of deoxyinosine on the incorporation of 5-FU into high molecular weight material

Addition	μM 5-FU	Cell line	
		WiDr	Intestine 407
RNA			
No	14	8.2 ± 0.4	7.20 ± 0.48
	33	19.3 ± 4.4	19.60 ± 3.38
0.4 mM Deoxyinosine	14	3.7 ± 0.4	2.98 ± 0.57
	33	6.6 ± 1.2	7.47 ± 1.17
1 mM Deoxyinosine	33	7.0 ± 1.0	10.20 ± 2.0
RNase stable			
No	14	0.59 ± 0.09	0.75 ± 0.06
	33	1.56 ± 0.35	1.45 ± 0.26
0.4 mM Deoxyinosine	14	0.84 ± 0.07	0.87 ± 0.23
	33	1.79 ± 0.66	1.33 ± 0.27
1 mM Deoxyinosine	33	1.61 ± 0.20	1.40 ± 0.32

Values (in pmol/h per 10^6 cells) are means ± S.E.M. of 3–4 separate experiments. Deoxyinosine was added simultaneously with 5-FU to the incubation mixture. Values of a 2 h incubation are shown.

the relatively low conversion of dIMP to deoxyinosine. A potentiation of 5-FU by GMP has been described for leukemic cells [17]. It is not likely that the intestinal cell lines WiDr and Intestine 407 show a potentiation of 5-FU by GMP since no potentiation by inosine was observed. The mechanism of potentiation by inosine would be the same as for GMP or its nucleoside guanosine.

The enhanced sensitivity to 5-FU induced by deoxyinosine can be attributed to the enhanced FdUMP formation [12, 31] and consequently enhanced binding of FdUMP to thymidylate synthase [31]. Kinetics of inhibition of thymidylate synthase were comparable in both cell lines and also comparable with literature data [36, 37]. However, not only is the extent of inhibition of thymidylate synthase important but also the retention of the inhibition [37–39]. This retention of inhibition is dependent on the FdUMP concentration and that of the folate [12, 38–41]. In the absence of precursors for FdUMP the recovery of thymidylate synthase was more pronounced, indicating the importance of a continuous availability of 5-FU in the culture medium [31]. The results with WiDr cells demonstrated that in this cell line inhibition of thymidylate synthase is not the only mechanism responsible for growth inhibition, even under conditions which favor FdUMP formation. Firstly, the retention of inhibition of thymidylate synthase does not correlate with the recovery in growth after 24 h up to 48 h. Furthermore, thymidine was not able to reverse growth inhibition by 5-FU and deoxyinosine between 0 and 24 h. It might be that in this cell line the low 5-FU incorporation into RNA still contributed to growth inhibition. It might also be possible that incorporation of 5-FU into DNA partly accounted for growth inhibition in this cell line [42].

The data in Table 6 for the RNase-stable material will be predominantly FdUMP bound to thymidylate synthase [30, 31]. Only a small portion might be attributed to incorporation into DNA. The results demonstrate that, although a higher activity of thymidylate synthase was observed in Intestine 407 cells, this was not accompanied by an enhanced binding of FdUMP to thymidylate synthase. This might be related with the more rapid recovery of thymidylate synthase after removal of 5-FU and deoxyinosine from the medium.

The results demonstrate that the use of deoxyinosine in the study of the mechanism of action of 5-FU can be useful. The inhibition of thymidylate synthase will be enhanced leading to enhanced cell growth inhibition. The retention of the inhibition partly correlated with the duration of growth inhibition. Future studies should be directed to enhance the retention of growth inhibition specifically in tumor cells in order to enhance the selectivity. The role of folates, either in the mono-glutamate form or as a polyglutamate, can be studied more in detail using the cell lines and the system described.

REFERENCES

1. Chabner BA. Pyrimidine antagonists. In: Chabner BA, ed. *Pharmacological Principles of Cancer Treatment.* Philadelphia, WB Saunders, 1982, 183–212.
2. Valeriote F, Santelli G. 5-Fluorouracil. *Pharmacol Ther* 1984, **24**, 107–132.
3. Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Sensitivity of human, murine and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res* 1986, **46**, 20–28.
4. Peters GJ, Laurensse E, Leyva A, Pinedo HM. The concentration of 5-phosphoribosyl-1-pyrophosphate in monolayer tumor cells and the effect of various pyrimidine antimetabolites. *Int J Biochem* 1985, **17**, 95–99.
5. Peters GJ, Laurensse E, Lankelma J, Leyva A, Pinedo HM. Separation of several 5-fluorouracil metabolites in various melanoma cell lines. Evidence for the synthesis of 5-fluorouracil nucleotide sugars. *Eur J Cancer Clin Oncol* 1984, **20**, 1425–1431.
6. Reyes P, Hall TC. Synthesis of 5-fluorouridine 5′-phosphate by a pyrimidine transferase of mammalian origin. II. Correlation between the tumor levels of the enzyme and 5-fluorouracil-promoted increase in survival of tumor bearing mice. *Biochem Pharmacol* 1969, **18**, 2587–2590.
7. Mandel HG. The target cell determinants of the antitumor actions of 5-FU: does 5-FU incorporation into RNA play a role? *Cancer Treat Rep* 1981, **65** (suppl. 3), 63–71.
8. Plagemann PGW, Wohlhueter RM. Permeation of nucleosides, nucleic acid bases and nucleotides in animal cells. *Curr Topics Membranes Transport* 1980, **14**, 225–330.
9. Gotto AM, Belkhode ML, Touster O. Stimulatory effects of inosine and deoxyinosine on the incorporation of uracil-2-^{14}C, 5-fluorouracil-2-^{14}C and 5-bromouracil-2-^{14}C into nucleic acids by Ehrlich ascites tumor cells *in vitro*. *Cancer Res* 1969, **29**, 807–811.
10. Kessel D, Hall TC. Influence of ribose donors on the action of 5-fluorouracil. *Cancer Res* 1969, **29**, 1749–1754.
11. Beltz RE, Waters RN, Hegarty TJ. Enhancement and depression by inosine of the growth inhibitory action of 5-fluorouracil on cultured Jensen tumor cells. *Biochem Biophys Res Comm* 1983, **112**, 235–241.
12. Evans RM, Laskin JD, Hakala MT. Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. *Cancer Res* 1981, **41**, 3288–3295.
13. Cory JG, Carter GL. Evidence that 5′-deoxy-5-fluorouridine may not be activated by the same mechanism as 5-fluorouracil. *Biochem Pharmacol* 1982, **31**, 2841–2844.
14. Cory JG, Breland JC, Carter GL. Effect of 5-fluorouracil on RNA metabolism in Novikoff hepatoma cells. *Cancer Res* 1979, **39**, 4905–4913.
15. Santelli G, Valeriote F. *In vivo* potentiation of 5-fluorouracil cytotoxicity against AKR leukemia by purines, pyrimidines, and their nucleosides and deoxynucleosides. *J Natl Cancer Inst* 1980, **64**, 69–73.
16. Ullman B, Kirsch J. Metabolism of 5-fluorouracil in cultured cells, protection from 5-fluorouracil cytotoxicity by purines. *Mol Pharmacol* 1978, **15**, 357–366.
17. Iigio M, Hoshi A. Influence of molar ratio on the combination effect of 5-fluorouracil with guanosine 5′-monophosphate on P388 and L1210 leukemias. *Eur J Cancer Clin Oncol* 1984, **29**, 411–415.
18. Piper AA, Fox RM. Biochemical basis for the differential sensitivity of human T- and B-lymphocyte lines to 5-fluorouracil. *Cancer Res* 1982, **42**, 3753–3760.
19. Ipata PL, Sgarrella F, Catalani R, Tozzi MG. Induction of phosphoribomutase in *Bacillus cereus* growing on nucleosides. *Biochim Biophys Acta* 1983, **755**, 253–256.
20. Peters GJ, Kraal I, Laurensse E, Leyva A, Pinedo HM. Separation of 5-fluorouracil and uracil by ion-pair reversed-phase high performance liquid chromatography on a column with porus polymeric packing. *J Chromatogr* 1984, **307**, 464–468.
21. Roberts D. An isotopic assay for thymidylate synthetase. *Biochemistry* 1966, **5**, 3546–3548.
22. Tax WJM, Veerkamp JH. A simple and sensitive method for estimating the concentration and synthesis of 5-phosphoribosyl 1-pyrophosphate in red blood cells. *Clin Chim Acta* 1977, **78**, 209–216.
23. Peters GJ, Veerkamp JH. Concentration, synthesis and utilization of phosphoribosyl-pyrophosphate in lymphocytes of various mammalian species. *Int J Biochem* 1979, **10**, 885–888.
24. Peters GJ, Laurensse E, Leyva A, Pinedo HM. Fluctuations in phosphoribosyl-pyrophosphate levels in monolayer tumor cell lines. Effect of drugs. *FEBS Lett* 1984, **170**, 277–280.
25. McIvor RS, Wohlhueter RM, Plagemann PGW. An improved enzymatic radioassay for ribose 1-phosphate. *Anal Biochem* 1982, **127**, 150–154.
26. Barankiewicz J, Henderson JF. Determination of ribose 1-phosphate in ascites tumor cells. *Biochem Med* 1977, **17**, 45–53.
27. Peters GJ, Oosterhof A, Veerkamp JH. Aenosine and deoxyadenosine metabolism in mammalian lymphocytes. *Int J Biochem* 1981, **13**, 445–455.
28. Guarino AJ, Sable HZ. Studies on phosphomutases. III. The phosphoribomutase of bovine uterus. *Biochim Biophys Acta* 1956. **20**, 201–208.
29. Barankiewicz J, Henderson JF. Ribose 1-phosphate metabolism in Ehrlich ascites tumor cells *in vitro*. *Biochim Biophys Acta* 1977, **479**, 371–377.
30. Parker WB, Klubes P. Enhancement by uridine of the anabolism of 5-fluorouracil in mouse

T-lymphoma (S-49) cells. *Cancer Res* 1986, **45**, 4249–4256.
31. Washtien WL. Comparison of 5-fluorouracil metabolism in two human gastrointestinal tumor cell lines. *Cancer Res* 1984, **44**, 909–914.
32. Parker WB, Kennedy KA, Klubes P. Dissociation of 5-fluorouracil-induced DNA fragmentation from either its incorporation into DNA or cytotoxicity in mouse T-lymphoma (S-49) cells. *Cancer Res* 1987, **47**, 979–982.
33. Moore MA, Ahmed F, Dunlap RB. Isolation of the covalent binary complex of 5-fluorodeoxyuridylate synthetase by trichloroacetic acid precipitation. *Biochim Biophys Res Comm* 1984, **124**, 34–37.
34. Yoshida M, Hoshi A. Mechanism of inhibition of phosphoribosylation of 5-fluorouracil by purines. *Biochem Pharmacol* 1984, **33**, 2863–2867.
35. Wice BM, Kennel DE. Sugar-free growth of mammalian cells on some ribonucleosides but not on others. *J Biol Chem* 1983, **258**, 13134–13140.
36. Danenberg PV. Thymidylate synthetase—a target enzyme in cancer chemotherapy. *Biochim Biophys Acta* 1977, **473**, 73–92.
37. Houghton JA, Torrance PM, Radparvar S, Williams LG, Houghton PJ. Binding of 5-fluorodeoxyuridylate to thymidylate synthase in human colon adenocarcinoma xenografts. *Eur J Cancer Clin Oncol* 1986, **22**, 505–510.
38. Houghton JA, Weiss KD, Williams LG, Torrance PM, Houghton PJ. Relationship between 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthase activity subsequent to 5-fluorouracil administration, in xenografts of human colon adenocarcinomas. *Biochem Pharmacol* 1986, **35**, 1351–1358.
39. Berger SH, Hakala MT. Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthase by 5-fluorouracil. *Mol Pharmacol* 1984, **25**, 303–309.
40. Waxman S, Bruckner H. The enhancement of 5-fluorouracil antimetabolic activity by leucovorin, menadione and α-tocopherol. *Eur J Cancer Clin Oncol* 1982, **18**, 685–692.
41. Yin M-B, Zakrzewski SF, Hakala MT. Relationship of cellular cofactor pools to the activity of 5-fluorouracil. *Mol Pharmacol* 1983, **23**, 190–197.
42. Lönn U, Lönn S. Interaction between 5-fluorouracil and DNA of human colon adenocarcinoma. *Cancer Res* 1984, **44**, 3414–3418.