MODULATION OF CYTOTOXICITY AND METABOLISM OF 5-FLUOROURACIL IN TWO INTESTINE CELL LINES

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INTRODUCTION

The antitumor activity of 5-fluorouracil (5FU) is dependent on its conversion to active nucleotides. The rate of direct conversion to FUMP, catalyzed by orotate phosphoribosyl transferase (OPRT), depends on the availability of the co-substrate phosphoribosylpyrophosphate (PRPP). The rate of the indirect conversion to FUMP or FdUMP via fluorouridine (FUR) or fluorodeoxyuridine (FUdR), respectively, catalyzed by a pyrimidine nucleoside phosphorylase (PNP), depends on the availability of the cosubstrates ribose-1-phosphate (Rib-1-P) and deoxyribose-1-phosphate (dRib-1-P). Nucleosides can act as ribose donors. Especially inosine and deoxyinosine are good sources since the major pathway of their metabolism involves phosphorolysis to hypoxanthine (1). Furthermore, these nucleosides are relatively non-toxic (2). In a variety of systems either inosine or deoxyinosine have been shown to increase growth inhibition by 5FU (3,4,5) and to enhance antitumor activity (6). It has also been shown that deoxyinosine or inosine can protect cells against 5FU (4,7).

The mechanism of the synergistic effect on 5FU cytotoxicity is different for inosine or deoxyinosine. Inosine enhances the incorporation of 5FU into RNA (5), while deoxyinosine can enhance the accumulation of FdUMP (3) leading to inhibition of thymidylate synthase. A lack of synergism with inosine and deoxyinosine can be due to interference by the nucleosides with 5FU anabolism leading to decreased 5FU incorporation into RNA (4). By studying 5FU metabolism, the inhibition of thymidylate synthase and the incorporation of 5FU into RNA can be quantitated, but the contribution of each parameter to cytotoxicity may remain unclear. The use of purine nucleosides as modulators of 5FU metabolism may give more insight into specific pathways of 5FU anabolism responsible for 5FU cytotoxicity. In order to examine these pathways we compared the effect of deoxyinosine on 5FU metabolism and cytotoxicity in two human colonic cell lines differing in their sensitivity to 5FU and 5'-deoxy-5-fluorouridine (Doxifluridine, 5'dFUR) a precursor of 5-fluorouracil.

MATERIALS AND METHODS

The origins of the cell lines, culture media, fetal bovine serum

have been described previously (8) as well as the culturing conditions and calculations for the growth inhibition experiments (9). For these experiments cells were grown in dialyzed serum. For the synergism studies nucleosides (or dIMP) and 5FU were added simultaneously to the cells. Cells were cultured for 24 and 48 hr after the addition of these compounds. The media in which the cells were cultured were collected after 24 and 48 hr, deproteinized and frozen until analysis. The concentrations of nucleosides and nucleotides were measured using standard HPLC methods, nucleosides on a RP-18 column and nucleotides on a Partisil SAX column.

The effect of nucleosides and dIMP on the PRPP concentration in cells was determined as described previously (10,11). The effect of nucleosides and dIMP on the concentration of the pentose phosphates was determined by a method analogous to that used for PRPP. The concentration of the pentose phosphates was measured by the method of McIvor et al. (12) with slight modifications.

All enzyme assays were performed using labeled 5FU as a substrate as described previously (9). Enzyme activities were measured with the co-substrates Rib-1-P and dRib-1-P and with the nucleoside precursors inosine and deoxyinosine. The activity of thymidylate synthase was measured at 1 and 10 µM dUMP as described (9). Measurement of the incorporation of tritiated 5FU into RNA and DNA was performed according to methods slightly modified from those described previously (8,9). Briefly, cells were precipitated on a filter with trichloroacetic acid. One half of the filters were counted directly, while the other half of the filters were incubated with RNAse to remove RNA. Thereafter, DNA was reprecipitated with trichloroacetic acid and the filters were counted.

RESULTS

Sensitivity to drugs of the WiDr and Intestine 407 cell lines was determined after 24 and 48 hr exposure to 5FU or the prodrug 5'dFUR. The IC 50 values after 24 hr exposure are summarized in Table 1. WiDr cells are more sensitive than Intestine 407 cells to 5FU as well as 5'dFUR. In order to study the effect of deoxyinosine on sensitivity to 5FU, low relatively non-toxic concentratons of 5FU were used in combination with increasing concentrations of deoxyinosine. Deoxyinosine itself did not inhibit cell growth at low concentrations, but at a high concentration growth inhibition was observed in WiDr cells and to a lesser extent in Intestine 407 cells (Fig. 1). At 10 µM deoxyinosine a synergism with 0.5 µM 5FU in WiDr and with 1.0 µM 5FU in Intestine 407 cells was found only after 24 hr exposure of the cells to the combination but not after 48 hr exposure. At higher concentrations of deoxyinosine a synergism was also

Table 1. Sensitivity of WiDr and Intestine 407 cells to 5FU and 5'dFUR.

Cell line	IC 50 (µM)	
	5FU	5'dFUR
Intestine 407	1.7 ± 0.6	82 ± 23
WiDr	0.7 ± 0.1	18 ± 5

Values are means ± SEM from 3-4 separate experiments. Cell number was determined after 24 hr exposure of the cells to the drugs, IC 50 values (the concentration that causes 50% growth inhibition) were calculated from the individual growth inhibition curves.

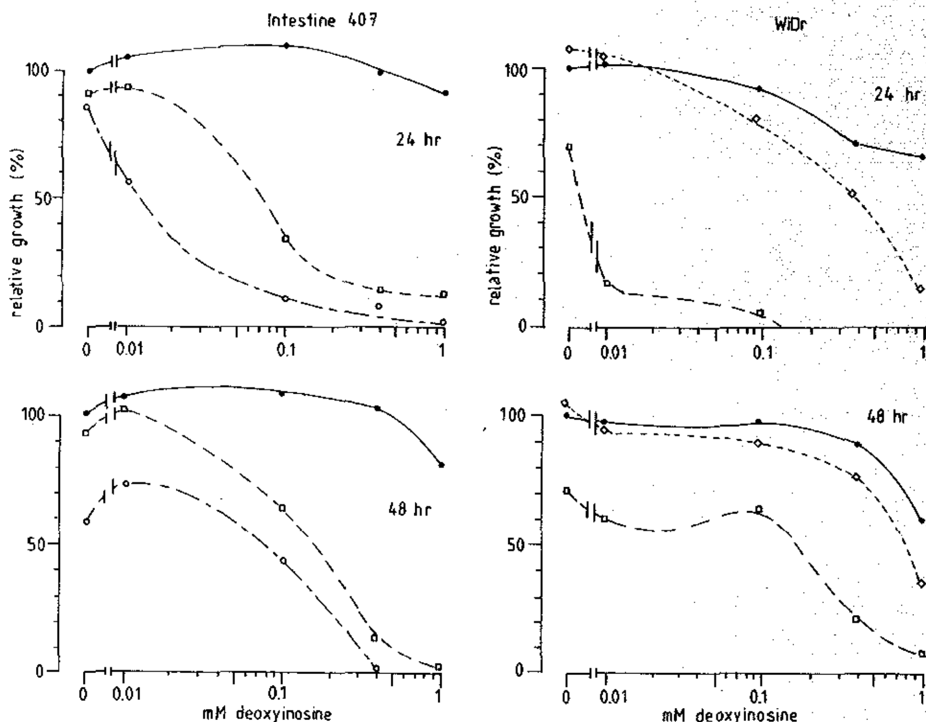

Fig. 1. Growth inhibition by 5FU in combination with deoxyinosine in Intestine 407 and WiDr cells. Values are means of 3-5 separate experiments. 5FU was present at 0.1 µM (◇---◇), 0.5 µM (□—-□) and 1 µM (o—-o) or not present (●——●).

observed with 0.1 µM 5FU in WiDr and with 0.5 µM in Intestine 407 cells. In both cell lines the synergistic effect of the combination appeared to be lower after 48 hr. This was more pronounced in the WiDr cells.

Synergistic studies were also performed with dIMP which can serve as a precursor for deoxyinosine after its dephosphorylation. No growth inhibition by dIMP was observed in either cell line. In Intestine 407 cells a marked synergism with the various combinations of 5FU and dIMP was observed (Fig. 2). The synergism was comparable to that observed with deoxyinosine. However, no synergism with dIMP was observed in WiDr cells (data not shown).

In order to investigate the mechanism of growth inhibition in both cell lines, thymidine was added to the cultures simultaneously with the combinations of 5FU with deoxyinosine and dIMP (Table 2). Thymidine prevented the growth inhibition by 5FU and deoxyinosine, and by 5FU and dIMP in Intestine 407 cells. However, this was not the case in WiDr cells. Thymidine even enhanced the inhibition by 5FU and deoxyinosine or dIMP.

Examination of the medium in which the cells were cultured in the presence of deoxyinosine demonstrated that in both cell lines a considerable part of deoxyinosine was phosphorolyzed to hypoxanthine (Table 3). After 48 hr more deoxyinosine appeared to be converted to hypoxanthine in Intestine 407 cells than in WiDr cells. Both cell lines

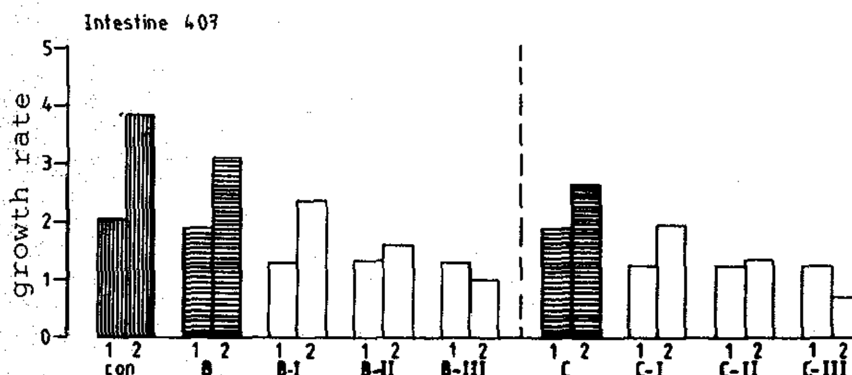

Fig. 2. Synergistic effect of 5FU and dIMP on growth of Intestine 407 cells. Growth rate was determined after one day (1) and two days (2). Bars are means of 2-4 separate experiments. Con, growth of control cells in the absence of any additions; B, 0.5 μM 5FU; C, 1.0 μM 5FU; I-III, 5FU and additional dIMP at 0.1, 0.4 and 1.0 mM, respectively.

Table 2. Effect of thymidine (TdR) on growth inhibition by 5FU in the presence of deoxyinosine or dIMP.

Cell line	Time (hr)	Control	5FU					
			No TdR		2 μM TdR		10 μM TdR	
			dino	dIMP	dino	dIMP	dino	dIMP
WiDr	24	1.76	1.09	1.42	1.23	1.35	0.92	0.9
	48	3.36	1.61	2.26	2.60	2.54	1.89	2.1
Intestine 407	24	1.96	1.19	1.27	1.46	1.75	1.71	1.7
	48	3.83	1.19	1.36	3.08	2.80	3.54	3.0

Growth rates are means of 3-6 separate experiments. In WiDr cells 5FU was present at 0.5 μM and in Intestine 407 cells at 1.0 μM. The concentration of deoxyinosine (dino) and dIMP was 0.4 mM. All compounds were added simultaneously to the cultures.

Table 3. Concentrations of metabolites of deoxyinosine and dIMP in culture medium of WiDr and Intestine 407 (I 407) cells.

Metabolite (μM)	Time (hr)	Addition (1 mM)			
		Deoxyinosine		dIMP	
		WiDr	I 407	WiDr	I 407
dIMP	24	-	-	647	650
	48	-	-	630	290
Deoxyinosine	24	750	727	64	198
	48	700	542	102	305
Hypoxanthine	24	243	249	73	144
	48	284	415	168	308
Inosine	24	6	22	-	-
	48	14	34	-	-

Table 4. Activity of PNP in WiDr and Intestine 407 cells with various co-substrates.

Co-substrate	WiDr	Intestine 407
Rib-1-P	1.3 ± 0.2	0.9 ± 0.1
Inosine	0.6 ± 0.2	0.85 ± 0.2
dRib-1-P	2.1 ± 0.3	1.3 ± 0.2
Deoxyinosine	0.1 ± 0.05	0.3 ± 0.13

Enzyme activities (in nmol/hr per 10^6 cells) are means ± SEM of 3-4 separate experiments and were measured with 5FU as substrate; the various co-substrates were present at 2.5 mM final concentration.

Table 5. Effect of inosine and deoxyinosine and dIMP on the concentration of Rib-1-P and dRib-1-P in WiDr and Intestine 407 (I 407).

Addition	Cell line	Rib-1-P	dRib-1-P
None	WiDr	0.72	-
	I 407	0.86	-
Inosine	WiDr	2.22	-
	I 407	2.54	-
Deoxyinosine	WiDr	0.72	1.80
	I 407	0.86	2.23
dIMP	WiDr	0.72	0.78
	I 407	0.86	2.97

Values (pmol/10^6 cells) are means of 3-5 separate experiments. Inosine, deoxyinosine and dIMP were present at 1 mM for 1 hr.

were able to convert hypoxanthine to inosine. Both WiDr and Intestine 407 cells converted dIMP to deoxyinosine and hypoxanthine. After 48 hr dephosphorylation was higher with the Intestine 407 cells than with WiDr cells. Inosine could not be detected under these circumstances.

The activities of enzymes responsible for 5FU activation were measured with 5FU as a substrate and with various co-substrates. The activity of OPRT, for which PRPP is the cosubstrate, was 2.07 in WiDr cells and 2.22 nmol/hr per 10^6 cells in Intestine 407 cells. The activity of PNP was slightly higher in the WiDr cell line (Table 4). PNP not only converts 5'dFUR to 5FU, but also 5FU to FUR and FUdR. PNP activity was also measured with inosine and deoxyinosine as precursors for the co-substrates Rib-1-P and dRib-1-P. In Intestine 407 cells PNP activity with inosine was comparable to that with Rib-1-P, but with deoxyinosine PNP activity was about 20% of that with dRib-1-P. In WiDr cells PNP activity with inosine and deoxyinosine was much lower than with Rib-1-P and dRib-1-P.

In both WiDr and Intestine 407 cells, inosine was able to increase the Rib-1-P concentrations 2- to 3-fold (Table 5). No dRib-1-P was detectable in untreated cells; after incubation with deoxyinosine the

Table 6. Inhibition of thymidylate synthase by FdUMP.

Cell line	FdUMP (μM)	Enzyme activity at 1 μM dUMP	Enzyme activity at 10 μM dUMP
WiDr	0	110 ± 14	380 ± 41
	0.01	16 ± 2	144 ± 17
	0.1	4 ± 2	28 ± 12
Intestine 407	0	430 ± 51	1358 ± 57
	0.01	39 ± 10	586 ± 25
	0.1	9 ± 2	116 ± 36

Values (in pmol/hr per 10^6 cells) are means ± SEM from 3-5 separate experiments.

concentration of dRib-1-P was comparable to that of Rib-1-P obtained after incubation with inosine. Incubation with dIMP resulted in much higher concentrations of dRib-1-P in Intestine 407 cells than in WiDr cells. In both cell lines the concentration of PRPP was reduced after a 2 hr incubation with inosine, deoxyinosine or dIMP (data not shown). Since the increase of the dRib-1-P pool due to deoxyinosine would result in an elevation of FdUMP levels (3) we measured the inhibition of thymidylate synthase by FdUMP (Table 6). Inhibition was measured at two concentrations of the substrate dUMP to examine for any kinetic differences between the two cell lines. The activity of thymidylate synthase was 3-4 times higher in Intestine 407 cells than in WiDr cells at both dUMP concentrations. The apparent Km value for dUMP was about 4 μM in both cell lines. The relative inhibition by 0.01 μM FdUMP was comparable in the two cell lines. At 0.1 μM FdUMP enzyme activity was inhibited more than 90% in both cell lines. The stimulation of 5FU anabolism towards deoxynucleotides produced at most a slight increase in the incorporation into DNA (Table 7). However, incorporation of 5FU into RNA was inhibited in both cell lines.

DISCUSSION

Modulation of the metabolism of 5FU with purine nucleosides can result in a specific enhancement of particular pathways of 5FU metabolism (3,5,13). This type of biochemical modulation will provide more information into the relative contribution of these pathways to the cytotoxicity of 5FU. Since inosine did not enhance the sensitivity to 5FU of WiDr and Intestine 407 cells (data not shown), we concentrated on the effect of deoxyinosine. Deoxyinosine appeared to be synergistic with 5FU, as was also demonstrated in other systems (3,6). Analogous to the similar effect of guanosine and GMP on potentiation of 5FU (13,14), the more soluble dIMP also potentiated the effect of 5FU. dIMP might also prevent a depletion in Pi which can occur when deoxyinosine is given as a precursor. The synergistic effect of dIMP and deoxyinosine with 5FU was comparable in Intestine 407 cells but not in WiDr cells. Analysis of the medium demonstrated that dephosphorylation of dIMP by WiDr is relatively low, which correlates with the lower ecto-phosphatase and ecto-nucleotidase activity in this cell line compared to Intestine 407 (9). As a consequence the expansion of the dRib-1-P pool by dIMP in WiDr cells is relatively low (Table 5). This means that less dRib-1-P is available to enhance the conversion of 5FU to FUdR and thus to FdUMP. This might account for the relatively little synergism found in WiDr cells for dIMP and 5FU.

Table 7. Effect of deoxyinosine on the incorporation of 5FU into RNA and DNA.

	WiDr		Intestine 407	
	RNA	DNA	RNA	DNA
Control	8.2	0.58	7.2	0.75
Deoxyinosine	3.7	0.84	3.0	0.87

Values (pmol/hr per 10^6 cells) are means of 2-3 experiments. Deoxyinosine (at 0.4 mM) was added simultaneously with 5FU to the incubation mixture.

The enhancement of sensitivity to 5FU by deoxyinosine indicates that inhibition of thymidylate synthase by FdUMP is an important mechanism for cytotoxicity of 5FU in both WiDr and Intestine 407 cells. FdUMP is a potent inhibitor of thymidylate synthase in both cell lines (Table 6) and is formed during incubation with 5FU (data not shown) and may inhibit thymidylate synthase in vivo. However, it is not clear whether the complex thymidylate synthase-FdUMP is stable in WiDr cells. It appears from the growth-inhibition experiments that inhibition is most pronounced at 24 hr but that between 24 and 48 hr growth rate is normal. The thymidylate synthase activity after 24 hr may be sufficient to support growth. In Intestine 407 the growth inhibition is more long-lasting since in the presence of 5FU and deoxyinosine or dIMP growth is still inhibited between 24 and 48 hr. This difference in the duration of growth inhibition might also account for the lack of reversal of growth inhibition by thymidine in WiDr cells. It is possible that WiDr only requires low amounts of dTMP to support growth since the activity of thymidylate synthase is relatively low in these cells. So a small recovery of thymidylate synthase would generate enough dTMP to support growth. Higher thymidine concentrations might even result in elevated dTTP levels which may lead in combination with purines to an enhanced growth inhibition (15).

The contribution to cytotoxicity from 5FU incorporation into DNA, which is even enhanced in WiDr cells by deoxyinosine is not clear. 5FU incorporated into DNA will be excised (16). The amount of 5FU which is incorporated into DNA may account for a steady state level; the actual amount of 5FU that has been incorporated into DNA will be higher. Fractionation of DNA of cells incubated with 5FU showed that lesions occur which have pronounced effects on both synthesis and stability of DNA (17). The higher amount of 5FU incorporated into DNA of WiDr in the presence of deoxyinosine may reflect a higher proportion of lesions and might be associated with toxicity. Since it may be possible that in the presence of thymidine these lesions will not be repaired, this would also account for the lack of reversal by thymidine of cytotoxicity.

In both cell lines 5FU is incorporated into RNA but deoxyinosine decreases this incorporation. Thus, incorporation of 5FU into RNA may have little or no impact on cells. However, in the presence of thymidine 5FU incorporation into RNA can be enhanced (18), which may also account for the lack of reversal of cytotoxicity of thymidine.

In conclusion, deoxyinosine increased the sensitivity to 5FU of WiDr and Intestine 407 cells. An acceleration of the metabolism of 5FU to FUdR and subsequently to FdUMP, supported by the increased dRib-1-P levels, appears to be responsible for this synergism. Inhibition of thymidylate synthase by FdUMP plays a major part in the cytotoxic ef-

fect of 5FU in both cell lines, but in WiDr cells an additional mechanism such as 5FU incorporation into RNA or DNA may also be responsible for the growth inhibition.

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