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Preclinical report

Modulation of 5-fluorouracil by 5-ethyl-2′-deoxyuridine on cell lines expressing different dihydropyrimidine dehydrogenase activities

Csilla Katona, Ferenc Tímár, András Jeney, Jean L Fischel,[1] Gerard Milano,[1] Erzsébet Pandi[2] and Judit Kralovánszky[2]

First Institute of Pathology and Experimental Cancer Reserch, Semmelweis University of Medicine, Budapest, Hungary. [1]Laboratory of Oncopharmacology, Centre Antoine Lacassagne, Nice, France. [2]National Institute of Oncology, Budapest, Hungary.

The purpose of the present study was to clarify the significance of the inhibition of dihydropyrimidine dehydrogenase (DPD) in the modulation of 5-fluorouracil (5-FU) action by 5-ethyl-2′-deoxyuridine (EUdR). Four human cell lines, which differed in their susceptibility to 5-FU and in their DPD activity, were selected as biological objects. Several other enzymes of pyrimidine metabolism, i.e. thymidylate synthase (TS), thymidine kinase (TK) and pyrimidine nucleoside phosphorylase (PNP), which might be involved in the 5-FU action were also studied to elucidate their potential role in the modulation of 5-FU cytotoxicity. Two out of the four cell lines, i.e. COLO1 and SW620, showed low (57 and 28 pmol/min/mg protein) and the other two cell lines, i.e. CAL51 and CAL33, showed high (235 and 184 pmol/min/mg protein) DPD activity, respectively. In our study, contrary to our expectation, no correlation between the DPD and TS activity of the cell lines and their 5-FU sensitivity could be observed. EUdR alone was cytotoxic only on CAL33 cells in a concentration below 1 mM (IC_{50}=194 μM) which might be due to the high TK activity (857 pmol/min/mg protein) measured in this cell line, favoring the formation of the phosphorylated nucleotides EdUMP and EdUTP indispensable for the inhibition of TS and DNA polymerase, respectively. Surprisingly, although EUdR by metabolizing to EUra was able to reduce the high activity of DPD in CAL33 and CAL51 cells by 47 and 55%, respectively, no potentiation of the 5-FU action occurred on these cell lines. On the contrary, enhancement of the 5-FU cytotoxicity was demonstrated on COLO1 and SW620 cells with low DPD activity. Our findings suggest that the 5-FU modulatory action of EUdR may be directed on other molecular targets than DPD as well, i.e. the augmentation of TS inhibition by EdUMP as demonstrated on SW620 cells might be one of these mechanisms. [© 1999 Lippincott Williams & Wilkins.]

Key words: 5-Fluorouracil modulation, 5-ethyl-2′-deoxyuridine, dihydropyrimidine dehydrogenase.

Introduction

5-Ethyl-2′-deoxyuridine (EUdR) is a potent antiviral agent which was synthesized in Hungary.[1] *In vitro* growth inhibition of EUdR was demonstrated against L5178Y and L1210 leukemic cells as well as Ehrlich ascites tumor cells;[2,3] however, it was ineffective against the same tumors *in vivo* tested up to 1000 mg/kg. EUdR is phosphorylated to EdUMP, EdUDP and EdUTP, and thus inhibits the activity of thymidylate synthase (TS) and could act as a substrate for DNA polymerase.[3,4] EUdR was shown to compete with thymidine for catabolism by thymidine phosphorylase from mouse liver and gut, forming 5-ethyluracil (EUra) and deoxyribose-1-phosphate.[3] In four human colorectal tumor xenografts EUdR potentiated the antitumor action of 5-fluorouracil (5-FU).[5] The complicated but rather well elucidated metabolism of 5-FU offers interesting possibilities for the biochemical modulation of its action. Several modulators of 5-FU metabolism such as leucovorin,[6] high-dose uridine,[7] PN-401, an oral prodrug of uridine,[8] that are already in clinical use or tested in clinical trials. It has been recently shown that the altered activity of the 5-FU catabolizing enzyme, dihydropyrimidine dehydrogenase (DPD), might be in connection with 5-FU-

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Correspondence to A Jeney, First Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine, Budapest, 1085 Üllöi str 26, Hungary.
Tel: (+36) 1-266-1638/4413; Fax: (+36) 1-317 1070;
E-mail: ajeney@korb1.sote.hu

associated toxicity and/or 5-FU sensitivity; consequently inhibition of DPD might be efficacious in increasing 5-FU activity.[9-12] There are several DPD inhibitors under investigation, e.g. ethynyluracil, an irreversible inactivator of DPD which enhanced the cytotoxic effect of 5-FU on cell lines expressing high DPD activity, while on cell lines with low DPD activity no similar effect was observed.[13] Ethynyluracil has already been tested in clinical phase I and pharmacokinetic studies.[14]

Recently, in our laboratory, a dose-dependent enhancement of the antitumor activity of 5-FU by EUdR on Colon-26 and Colon-38 tumors was at least partly assigned to the inhibition of DPD by EUra, the metabolite of EUdR.[15] As a consequence of DPD inhibition, the C_{max} and AUC values of 5-dihydrofluorouracil (H_2FU), i.e. the first catabolite of 5-FU, decreased. DPD isolated from mouse liver was inhibited by EUra dose dependently between 10 and 100 μM, and the calculated K_i value was found to be 13.6 μM.[15] EUdR was also found to inhibit the amount of $^{14}CO_2$ formed from [2-^{14}C]5-FU and expired within 8 h, in mice treated by the labeled 5-FU and EUdR.[16] Based on these data, the purpose of the present study was to investigate the effect of EUdR and EUra on 5-FU sensitivity of four cell lines expressing different DPD activities, and thus to find out whether inhibition of this enzyme is a determinant factor in the enhancement of 5-FU cytotoxicity by EUdR.

Materials and methods

Chemicals

EUdR and EUra were obtained from the Central Chemical Research Institute of the Hungarian Academy of Sciences (Professor Dr László Ötvös). 5-FU was purchased from Hoffman La Roche (Basle, Switzerland). Culture media, MTT and all other chemicals were obtained from Sigma (St Louis, MO).

Cell lines

Four human cancer cell lines, i.e. two colon (COLO1 and SW620), one head and neck (CAL33), and one mammary carcinoma (CAL51) line, were used. Three of these cell lines (COLO1, CAL51 and CAL33) were kindly provided by the Laboratory of Oncopharmacology, Centre Antoine Lacassagne, Nice, France, and stored in liquid nitrogen until use. The cell lines were maintained as a monolayer in DMEM (CAL33, CAL51 and SW620) and in RPMI 1640 (COLO1) medium supplemented by 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

Determination of DPD, TS, thymidine kinase (TK) and pyrimidine nucleoside phosphorylase (PNP) activities of the cell lines

For all of the above-mentioned enzyme assays the cells were harvested in the exponential phase, washed 3 times in PBS and counted by a hemocytometer, and finally a cell suspension of 10^7 cells/ml in PBS containing 10% glycerol was prepared. On the day of the assay, the cell suspension was freeze-thawed 3 times and then centrifuged for 30 min at 28 000 r.p.m., i.e. 100 000 *g*. The cytosol was then used for the different enzyme assays immediately.

DPD. DPD activity was determined by the method of Naguib[17] using [6-^{14}C] 5-FU (Amersham, Little Chalfont, UK; 54 mCi/mmol) as a substrate. The cytosol was incubated with [6-^{14}C]5-FU (26.5 μM final), NADPH (120 μM final), magnesium chloride (6.5 mM final) and nicotinamide (1.0 μM final) in sodium phosphate buffer (pH 8.0) at 37°C for 30 min (total volume was 200 μl). The reaction was stopped by adding 200 μl of ice-cold ethanol. The incubation mixture was kept on ice for 30 min, and then it was filtered in a Whatman polypropylene microfilter (0.45 μM) and centrifuged (4000 r.p.m., 15 min). Samples were stored at −20°C until the HPLC analysis. The catabolite H_2FU was separated from the substrate 5-FU by the HPLC technique with a Merck Hitachi LichroGraph HPLC system according to Sommadossi *et al.*[18] The radioactivity of the catabolite was measured by a Beckmann liquid scintillation spectrometer. DPD activities were expressed as the amount of H_2FU formed per minute per mg cytosolic protein (pmol/min/mg protein).

TK. Cytosolic TK activites were measured according to Klemperer and Haynes[19] by determining the formation of dTMP from [2-^{14}C]thymidine (Nycom Prague, Czech Republic; 53.6 mCi/mmol). The cytosol was incubated with [2-^{14}C]thymidine (332 μM final), ATP (4.16 mM final), magnesium chloride (2.0 mM final) in Tris buffer (pH 8.0) (16.5 mM final) in a total volume of 600 μl. The incubation continued for 15 and 30 min at 37°C, and was stopped by dropping 50 μl of the incubation mixture on DEAE cellulose disks (ion exchange paper DE81; Whatman, Maidstone, UK) absorbing thymidine monophosphate only

and washed in ammonium formate solution (1 mM) and then in distilled water to eliminate the non-converted thymidine. After drying, the radioactivity of the disks was measured by a Beckmann liquid scintillation spectrometer. TK activity was expressed as the amount of thymidine mono-phosphate formed per minute per mg protein (pmol dTMP/min/mg protein.).

TS. TS activity was determined according the ^{3}H-release assay described by Roberts[20] using [5-^{3}H]dUMP (Amersham; 15.4 mCi/mmol) as substrate. The assay consisted of Tris buffer (pH 7.5) (1.6 mM), sodium fluoride (16.0 mM), [5-^{3}H]dUMP (0.3 and 3 M), 5,10-methylene-tetrahydrofolate (70 μM) in a total volume of 525 μl. Reaction was started with 100 μl of cytosol. After 15 and 30 min incubation at 37°C the reaction was stopped with 3.5% TCA and the excess of [5-^{3}H]dUMP was removed by activated charcoal. After centrifugation radioactivity was measured from the supernatant with a Beckmann liquid scintillation spectrometer. TS activity was expressed as the amount of thymidine monophosphate formed per minute per mg protein (pmol dTMP/min/mg protein).

PNP. PNP activity in the cell lines was determined by measuring the formation of [^{14}C]thymine from its respective 2-^{14}C-labeled nucleoside {[2-^{14}C]thymidine (Nycom; 53.6 mCi/mmol)} according to the method described by Ashour.[21] The assay consisted of phosphate buffer (pH 8.0) containing EDTA (1 mM) and dithiothreitol (1 mM), [2-^{14}C]thymidine (500 μM) in a total volume of 200 μl. The reaction was initiated by adding 100 μl of cytosol. The product was separated from the substrate on thin-layer plates (silicagel TLC plates, Merck, Darmstadt, Germany; Alufolien Kieselgel 60 F 254) developed in chloroform:methanol:acetic acid 90:5:5 v/v/v. Radioactivity of the spots was measured by a Beckman liquid scintillation counter. PNP activity was expressed as the amount of thymine formed per minute per mg protein (pmol/min/mg protein).

Chemosensitivity tests

Exponentially growing cells were distributed in 96-well microtitration plates at an initial cell density of 5×10^{3} cells/well. The cells were then exposed to either EUdR or EUra for 30 min, and then to 5-FU for another 4 h, when the culture medium was changed. Although the recently published meta analysis showed a slight increase in tumor response and overall survival in patients treated by continuous infusion of 5-FU,[22] since our purpose was to increase the cytotoxic action of 5-FU a treatment schedule was selected which could make possible the further improvement of the cytotoxic activity of 5-FU. Similar to the method used by Fischel[13] and Longo,[23] the 4 h exposure was chosen to mimic the bolus 5-FU administration. The cytotoxic effect of 5-FU, 5-FU+EUdR and 5-FU+EUra was assessed after 120 h incubation using the MTT test. The absorbance of each well was measured at 540 nm using a microtiter plate reader (Multiscan MS; Labsystems, Helsinki, Finland), the blank was considered as '0' reading and was adjusted by the reader automatically. The rate of growth inhibition was expressed as the percentage of controls without drug. Dose-response curves and IC_{50} values were calculated with the help of the computer program GraphPad Prism. The 5-FU modulatory activity of EUdR and EUra was characterized by the increase of the enhancement factors calculated as follows: EF=IC_{50} 5-FU/IC_{50} [EUdR+5-FU]; IC_{50} FU/IC_{50} [EUra+5-FU].

Statistical analysis was performed with the help of the computer program 'Statist' based on Tallarida and Murray.[24]

Results

The principle question addressed in this study was whether a correlation exists between the activity of DPD and the modulatory capacity of EUdR on 5-FU cytotoxicity. Therefore two cell lines with high and two with low DPD activities were selected to determine the antiproliferative action of 5-FU and EUdR combinations. Moreover, the activities of various enzymes known to be involved directly or indirectly in the metabolism of 5-FU were supposed to provide further information to elucidate their potential role in the modulation of 5-FU action by EUdR. Our data concerning the activities of DPD, TK, TS and PNP in the four studied cell lines are summarized in Table 1.

The results show that the activity of DPD as the catabolic enzyme for 5-FU was high in the CAL51 and CAL33 cells (i.e. 235 and 184 pmol/min/mg protein) and relatively low in COLO1 and SW620 cells (i.e. 57 and 28 pmol/min/mg protein), respectively. The value of the salvage pyrimidine biosynthetic enzyme TK was approximately 2-3 orders of magnitude higher in all studied cell lines (range: 390-857 pmol/min/mg protein), compared to the activity of TS, and the highest value, 857 pmol/min/mg protein, was measured in the CAL33 cells. In the literature different protocols were described for the measurement of TS activity. In these methods the concentration of the substrate dUMP changed from 0.04 to 100 μM.[25,26] Previously the TS activity had been measured on several cell lines

Table 1. The activity of the pyrimidine metabolic enzymes of the investigated cell lines

Cell line	Enzyme activity[a]				
	DPD	TK	TS_1	TS_2	PNP
CAL33	184.6±19.1 (3)	857.0±350.8 (4)	0.38±0.10 (3)	2.4±0.35 (3)	4247.0±1248.0 (3)
CAL51	235.0±29.6 (3)	565.5±125.9 (4)	1.90±0.78 (3)	10.6±4.6 (3)	414.5±115.5 (3)
COLO1	57.8±5.3 (5)	526.5±169.4 (4)	0.67±0.08 (3)	3.4±1.2 (3)	287.0±93.0 (3)
SW620	28.2±7.2 (6)	390.2±25.4 (5)	0.34±0.07 (3)	2.4±1.0 (3)	256.5±5.5 (3)

[a]Enzyme activities pmol/min/mg protein mean values±SD are given.
Values in parentheses indicate number of determinations.
TS_1 activity was measured at 0.3 μM and TS_2 at 3.0 μM dUMP concentration, respectively.

Table 2. Effect of EUdR and EUra on the cytotoxic action of 5-FU

Cell line	IC50 (μM)[a]		5-FU enhancement factor (EF)[b]			
	5-FU	EUdR	EUdR			EUra
			1.0 μM	10 μM	100 μM	100 μM
CAL33	108±64	194±53.1	1.07	0.98	0.90	1.01
CAL51	210±36	>1000	0.88	0.92	1.05	2.27
COLO1	139±51	1000±280	0.98	1.11	2.43	0.97
SW620	252±42	>1000	1.49	2.27	2.50	1.11

[a]IC_{50} mean value±SD of three to five measurements.
[b]Enhancement factors were calculated as given in the text.

(CAL33, CAL51 and CAL27) and the K_m value of the dUMP had been found to be between 0.46 and 0.53 μM.[27] In the present study TS activity was measured at 0.3 μM dUMP which is around the K_m value (TS_1) and at 3.0 μM (TS_2). The TS_1 activity varied from 0.34 to 1.9 and that of TS_2 from 2.4 to 10.6 pmol/min/mg protein, respectively. The ratio between TS_2/TS_1 changed from 5.1 to 7.0. The highest TS_2 value (10.6 pmol/min/mg protein) was measured in CAL51 cells. The value of PNP varied from 256 up to 4274 pmol/min/mg protein in the cells and surprisingly its activity was about 10- to 16-fold higher in CAL33 compared to the other three cell lines.

In order to analyze the relationship between the DPD expression of the different cells and their 5-FU sensitivity, the change of the IC_{50} value of 5-FU was compared on the various cell lines. Table 2 indicates that 5-FU inhibited the cell proliferation on each cell line, its IC_{50} values changed between 108 and 252 μM; however, no correlation could be demonstrated between the IC_{50} values of 5-FU and the DPD activity of the cells.

The growth inhibitory effect of EUdR differed on the various cell lines. On CAL33 cells EUdR was cytotoxic itself (IC_{50}=194 μM); on the contrary, its IC_{50} value was relatively high (1000 μM) on COLO1 cells. The same concentration resulted only in a moderate (30%) cytotoxicity on the CAL51 cell line and the SW620 cells were insensitive against EUdR, tested up to 1000 μM. It is noteworthy that up to 100 μM EUdR showed no antiproliferative activity in any of the cell lines included in this study.

Figure 1(A) shows that the cytotoxic action of 5-FU was potentiated by EUdR in the two cell lines with low DPD activity (SW620 and COLO1). In SW620 cells the values of the enhancement factors increased, in a concentration-dependent manner, from 1.49 up to 2.5 (Table 2), indicating the most efficacious modulation of 5-FU at 100 μM EUdR. The enhancement of the 5-FU activity by EUdR on COLO1 cell line was registered only at 100 μM EUdR, resulting in an enhancement factor of 2.43 (Figure 1B and Table 2). No increase of 5-FU action by EUdR could be observed on CAL33 and CAL51 cells, the enhancement factors varied around 1 (Table 2).

EUra (the main metabolite of EUdR), applied alone up to 100 μM, showed no cytotoxicity against any of the cell lines examined, therefore its IC_{50} value could not be calculated (data are not shown). Unfortunately the solubility of this compound did not allow us to use higher concentrations. Out of the two cell lines with

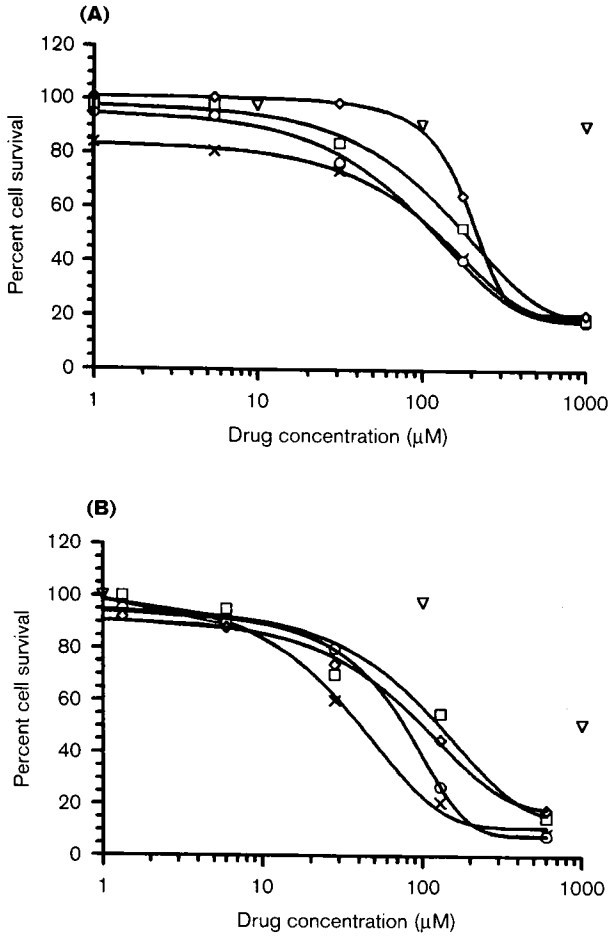

Figure 1. The effect of EUdR 5-FU cytotoxicity on SW620 (A) and COLO1 (B) cell lines (percentage of cell survival compared to controls without drug, as a function of drug concentration in the culture medium): (▽) EUdR alone, (◇) 5-FU alone, (□) 5-FU+1 μM EUdR, (○) 5-FU+10 μM EUdR and (X) 5-FU+100 μM EUdR.

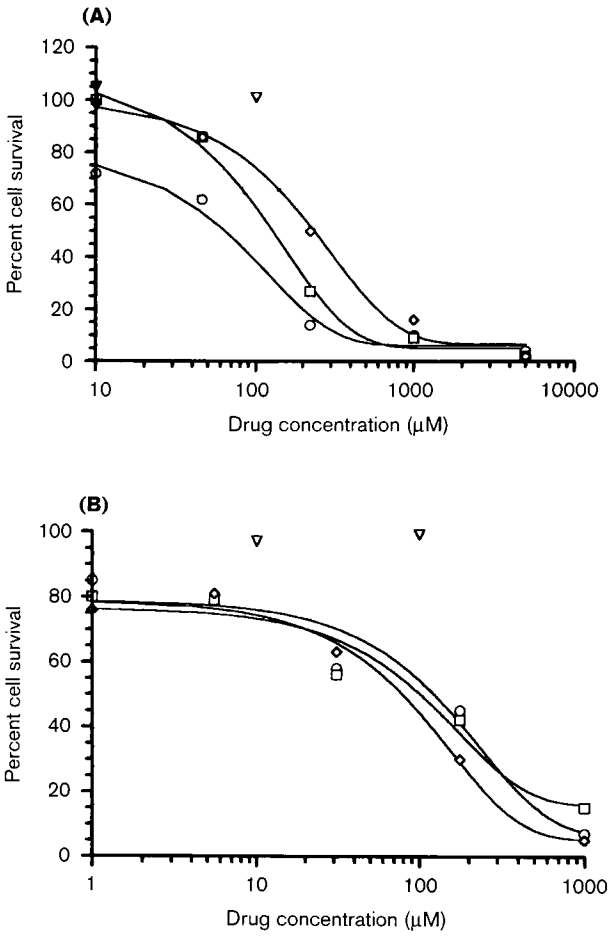

Figure 2. The effect of EUra on 5-FU cytotoxicity on CAL51 (A) and CAL33 (B) cell lines (percentage of cell survival compared to controls without drug, as a function of drug concentration in the culture medium): (▽) EUra alone, (◇) 5-FU alone, (□) 5-FU+10 μM EUra and (○) 5-FU+100 μM EUra.

high DPD activity, EUra potentiated the cytotoxic effect of 5-FU on CAL51 cells resulting in an enhancement factor of 2.27 (Figure 2A and Table 2), whereas on the other cell line (CAL33), although it has also high DPD activity, the combination of EUra with 5-FU did not result in a more extensive cytotoxicity. (Figure 2B and Table 2).

Similarily there was no change in the cytotoxic action of 5-FU in the presence of EUra in other cell lines expressing low DPD activity.

Our studies on the inhibition of DPD activity by EUdR and EUra on the two cell lines with high DPD activity showed that a substantial 91.5 and 97.5% reduction of the DPD activity could be observed in the CAL33 and CAL51 cell cultures, respectively, after applying 100 μM EUra. Using the same concentration of EUdR (100 μM) the DPD activity was inhibited by 46.9 and 54.6% in the CAL33 and CAL51 cells, respectively.

Discussion

In recent years DPD has been found to be the key enzyme in the catabolic pathway of 5-FU.[9,28] Subsequently a correlation between low DPD activity in normal cells, such as lymphocytes, and the organ toxicity in patients treated with 5-FU was concluded in several studies.[12,29,30] At the same time a significant effort was concentrated on the identification of DPD inhibitors to enhance the antitumor action of 5-FU and ethynyluracil was selected as the most promising

agent.[11] Since our previous studies showed that EUdR could enhance the antitumor action of 5-FU both in murine and human *in vivo* tumor model systems,[5,15] it was decided to compare the cytotoxic action of 5-FU without and after pretreatment with EUdR in cell lines, showing high and low DPD activity.

The first question addressed was whether the cytotoxicity of 5-FU alone is dependent on the level of DPD activity. Surprisingly contrary to our expectation no correlation between the DPD activity of the cell lines and their 5-FU sensitivity could be observed. Moreover we also failed to demonstrate an association between TS activity and 5-FU sensitivity. Thus these data could not confirm the reports indicating that the cell lines most sensitive to 5-FU exhibited the lowest DPD and TS activity.[13,27] In the study of Beck *et al.*[27] on a relatively large panel of human tumor cell lines (six breast, eight digestive tract, and five head and neck) both TS and DPD activities proved to be independent and complementary markers of 5-FU sensitivity, although the regression analysis showed only a moderate relationship; r^2 for TS was 0.22 and that for DPD was 0.27, respectively. A similar relationship (r^2=0.27) for TS was observed by Peters *et al.*[31] in a panel of 14 cell lines with different histological origins. However, in a more recent study analyzing the pharmacobiochemical determinants of 5-FU action on six different cell lines, among them the CAL51 and CAL33 cell lines also included in the present study, no significant correlation was established between the DPD or TS activity and the cytotoxic action of 5-FU.[32]

As far as the cytotoxicity of EUdR is concerned at concentrations below 1 mM, out of the four cell lines only the growth of CAL33 cells was inhibited (IC_{50}=194 μM). Perhaps the dedicated sensitivity is due to the fact that activity of TK was the highest in the CAL33 cell line which favors the formation of the phosphorylated nucleotides, EdUMP and EdUTP, indispensable for the inhibition of TS and DNA polymerase, respectively.[3] Although TS activity in the SW620 cell culture could be inhibited by 46% with 100 μM EUdR this did not result in cytotoxicity (data not shown).

In the present study we intended to clarify whether EUdR, similar to the results of the *in vivo* experiments, is able to increase the cytotoxic action of 5-FU in tissue culture and if there is any relationship with the level of DPD activity. The relevance of this assumption has been supported by the data indicating that DPD activity of CAL33 and CAL51 cells in the presence of 100 μM EUdR could be inhibited by 47 and 55%, respectively. The expectations were only partially fulfilled because EUdR did enhance the cytotoxic action of 5-FU in the two cell lines with low DPD activity (COLO1 and SW620) while no effect on the 5-FU action could be demonstrated on the cells with high DPD activity. In CAL33 cells EUdR is either phosphorylated by TK or, due to the high PNP activity, is degraded rapidly to EUra and deoxyribose-1-phosphate, which could be used for the formation of 5-fluoro-5′-deoxyuridine, the immediate precursor of FdUMP, the direct inhibitor of TS. This reaction normally does not occur in the cells due to the limited amount of the available deoxyribose-1-phosphate. The lack of modulation of 5-FU by EUdR apparently means that PNP does not metabolize 5-FU in this cell line even when more deoxyribose-1-phosphate is present or the inhibition of the *de novo* pyrimidine biosynthesis leads to even higher salvage activity, an efficient mechanism which could allow the cells to escape from TS inhibition.

As a further interest EUra, the major metabolite of EUdR, which produced a considerable reduction of the high DPD activity in both CAL33 and CAL51 cells, enhanced the cytotoxic action of 5-FU only in the CAL51 cell line, which likely means that in addition to DPD inhibition other characteristics of pyrimidine metabolism of the different cell lines, i.e. competing normal substrates, might influence the modulation of 5-FU cytotoxicity. On the contrary, ethynyluracil (776C), an irreversible inhibitor of DPD,[33,34] increased the 5-FU sensitivity in cell lines exhibiting high DPD activity.[13] The difference between ethynyluracil and EUra in terms of their 5-FU modulatory action may be due to their various chemical interactions with DPD. Baccanari *et al.*[34] reported that ethynyluracil binds irreversibily by covalent linkage to DPD which could explain the substantial higher 5-FU toxicity both *in vitro* and *in vivo*.

As a conclusion of this study it seems worthwhile to emphasize that DPD or TS activity cannot be utilized as a predictor for the cell growth inhibitory action of 5-FU although measurements on DPD activity in lymphocytes could provide important data to predict the organ toxicity of 5-FU in clinical situations. Since in our present study no indications for DPD involvement in the modulatory action of EUdR could be concluded, at least in the *in vitro* system, it is highly probable that other mechanisms may be responsible for the enhancement of 5-FU cytotoxicity. It is our forthcoming task to test the augmentation of the damage in the molecular targets of 5-FU, i.e. the dynamics of inhibition of TS activity and RNA synthesis.

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