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RETENTION OF *IN VIVO* ANTIPYRIMIDINE EFFECTS OF BREQUINAR SODIUM (DUP-785; NSC 368390) IN MURINE LIVER, BONE MARROW AND COLON CANCER

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Abstract—Brequinar sodium (DUP-785) is a potent inhibitor of the pyrimidine *de novo* enzyme, dihydroorotic acid dehydrogenase (DHO-DH). In order to determine whether *in vitro* data could be extrapolated to the *in vivo* situation we investigated antipyrimidine effects of DUP-785 in mice bearing colon cancer. Two tumor models were used, Colon 26 and Colon 38, resistant and moderately sensitive to DUP-785, respectively. DUP-785 at 50 mg/kg caused a depletion of plasma uridine in mice, and depleted tissue uridine levels in Colon 38 down to 10%, which was retained for several days; in Colon 26 the decrease was less and tissue uridine levels recovered rapidly. In livers of these mice no significant effect on uridine was observed. DUP-785 depleted UTP in bone marrow cells within 2 hr to 25% of control levels, after 4 days normal levels were found. In livers of both Balb-c mice (bearing Colon 26) and C57Bl/6 mice (bearing Colon 38) a small decrease of uridine nucleotide pools was found. In Colon 26 DUP-785 increased uridine nucleotide pools to 170% after 2 hr, at 1 day normal levels were observed, but after 2 days again an increase was found. In Colon 38 DUP-785 decreased the uridine nucleotide pool by 50% after 1 and 2 days. DUP-785 did not affect cytidine nucleotide pools of livers and of Colon 26 and Colon 38. The ratio between uridine nucleotides and cytidine nucleotides decreased from 2.2 to 0.90 in Colon 38, in the other tissues the decrease was less.

DHO-DH was measured in bone marrow cells and Colon 26 and 38 before and after treatment. Basal levels of DHO-DH were 3 times higher in Colon 26 than in Colon 38. In treated tumors DHO-DH was initially inhibited by more than 90%, after 7 days enzyme activity in Colon 26 was 50% and in Colon 38 about 200% of basal levels. In bone marrow cells DHO-DH was also rapidly inhibited but recovered within 4 days. It is concluded that the retention of antipyrimidine effects of DUP-785 in Colon 38 were more pronounced than in Colon 26, which is in agreement with the better antitumor effect of DUP-785 in Colon 38.

Dihydroorotic acid dehydrogenase (DHO-DH)‡ is a unique enzyme in the *de novo* synthesis of pyrimidine nucleotides. It is the fourth enzyme in the *de novo* pathway, and unlike the other enzymes it is not a part of an enzyme complex, nor located in the cytoplasma. Its location is on the outer side of the inner membrane of the mitochondrion [1, 2]. The first three enzymes, CPS II, ATC and DHOase, form an enzyme complex [3] as well as OPRT and ODC [3]. Several inhibitors of CPS II, ATC and the OPRT–ODC complex have been evaluated as anticancer agents and tested in Phase I and II trials [4–8]. Although PALA, an inhibitor of ATC, showed considerable activity in murine solid tumors [4], it was inactive against human solid tumors [8]. The same holds for pyrazofurin, an inhibitor of OPRT-ODC [5]. Acivicin, a glutamine-analog, is not only an inhibitor of CPS II, but also of other glutamine requiring enzymes, such as CTP synthetase and the purine enzyme GMP synthetase [6, 7]. Both PALA and acivicin are also being evaluated as a modulating agent [8, 9]. For PALA and acivicin, it has been demonstrated that comparable antipyrimidine effects can be observed both *in vivo* and *in vitro* [7, 10].

Recently it was demonstrated independently by Chen *et al.* [11] and our group [12] that DUP-785 is a potent inhibitor of DHO-DH, with a very low K_i of 20–100 nM. *In vitro* treatment of cells with DUP-785 led to a depletion of both pyrimidine ribo- and deoxyribonucleotides, but not of purine nucleotides [13]. The depletion of dTTP and dCTP was proportional to that of UTP and CTP and could be prevented by treatment of cells with uridine [13]. Growth-inhibition by DUP-785 could be reversed by uridine and cytidine, but not by the deoxynucleosides thymidine and deoxycytidine leading to the conclusion that inhibition of UMP synthesis is crucial for the *in vitro* activity of DUP-785 [12, 13]. Depletion of the pyrimidine nucleotides in L1210 cells was accompanied by inhibition of DHO-DH [13]. DUP-785 has now been evaluated in several Phase I trials. In the 3-weekly schedule we studied antipyrimidine effects of DUP-785 *in vivo* and observed a depletion of plasma uridine followed by a rebound, DHO-DH in lymphocytes of these patients was not detectable after treatment [14]. The extent of the effects on uridine and retention of DHO-DH appeared to correlate with toxicity.

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‡ Abbreviations: CPS-II, carbamyl-phosphate synthetase II; ATC, aspartate transcarbamylase; DHOase, dihydroorotase; DHO-DH, dihydroorotate dehydrogenase; OPRT, orotic acid phosphoribosyltransferase; ODC, OMP decarboxylase; PALA, *N*-(phosphon)acetyl-L-aspartate; L-DHO, L-dihydroorotate; TCA, trichloroacetic acid; PBS, phosphate buffered saline.

For proper evaluation of the mechanism of action of DUP-785 *in vivo* and interpretation of clinical data, analysis of the antipyrimidine effects in the target tissues is required. We used two murine colon cancers as model systems and studied the effects of DUP-785 on tumor growth, on pools of uridine and pyrimidine nucleotides and on retention of DHO-DH inhibition. Since bone marrow depression was dose-limiting in the Phase I trial we also studied enzyme inhibition and nucleotide levels in this tissue, while nucleotides and uridine were also measured in livers, for which no toxicity has been reported.

MATERIALS AND METHODS

Materials. DUP-785 (Brequinar sodium, NSC 368390) was synthesized and obtained from the Medicinal Chemistry Section, DuPont Pharmaceuticals, Wilmington, Delaware, U.S.A. It was formulated as a 10 mg/ml solution in saline. L-Dihydroorotic acid, orotic acid, uridine and nucleotides were obtained from Sigma Chemical Co., Ohio. Other compounds were of analytical grade quality. A prepacked LiChrosorb 5-RP-18 column (150 × 4.6 mm, length × i.d.; particle size 5 μm) and a prepacked Partisil-SAX column (250 × 4.6 mm, length × i.d.; particle size 10 μm) were obtained from Chrompack, Middelburg, The Netherlands.

Treatment of mice. Female C57Bl/6 and Balb-c mice were obtained at 6–8 weeks of age from the animal breeding station "Proefdieren-bedrijf TNO", Zeist, The Netherlands. They were kept in an area with standardized light–dark cycle for at least 10–14 days prior to the beginning of an experiment. Mice had access to food and water *ad lib.* Colon 26 and Colon 38 are murine colon adenocarcinomas maintained in Balb/c mice and C57Bl/6 mice, respectively. Their sources and growth characteristics have been described previously [15, 16]. At an age between 2 and 3 months tumors were transplanted as 1–5 mm^3 fragments subcutaneously in the flanks of the animals. Growth of the tumors was determined by caliper measurement (length × width × thickness × 0.5) once to twice a week. Treatment was started when tumor volume was between 50 and 250 mm^3 (19 days after transplantation for Colon 38 and 10 days for Colon 26). The volume of the tumors was calculated relative to that of the first day of treatment (day 0). Before treatment mice were randomized in two groups of each six mice, one control group and one receiving 50 mg DUP-785/kg as i.p. injections at days 0, 4, 8 and 12. The antitumor effect was evaluated by using the T/C (volume of treated tumors divided by that of control tumors) and the growth delay factor [15]: GDF = $(TD_{TR} - TD_c)/TD_c$, where TD_{TR} is the tumor doubling time of tumors from treated mice and TD_c that from untreated mice.

Measurement of nucleotides and uridine in tissues. The effect of DUP-785 on the levels of pyrimidine nucleotides and uridine was determined after a single i.p. injection of DUP-785 at 50 mg/kg. Mice were killed by cervical dislocation; tumors and livers were removed immediately and directly frozen in liquid nitrogen as described previously [17].

For analysis the frozen tissue sections were weighed and subsequently pulverized using a microdismembrator [17, 18]. The powder (still frozen) was extracted with a TCA solution (final concentration 5%) for 20 min at 4°. The denatured protein was spun down and the supernatant neutralized with 2 vol. alamine–Freon [19]. Part of the aqueous phase was used for the measurement of uridine. Analysis of total pyrimidine nucleotide pools (UΣP and CΣP) was performed by addition of perchloric acid to the other part to a final concentration of 1 M and followed by heating at 100° for 14 min. This procedure hydrolyses UTP, UDP and UDP sugars to UMP, and CTP, CDP and CDP sugars to CMP [10, 17, 20]. The hydrolysed extract had to be neutralized with 3 × 5 vol. of alamine–Freon because of the high concentration of perchloric acid.

Tissue uridine was determined with reversed phase HPLC using a LiChrosorb 5-RP-18 column. The column was eluted with 5 mM KH_2PO_4, pH 6.8 containing 5% methanol at a flow of 1 ml/min. Uridine was quantified by measurement of UV absorption at 280 and 254 nm and by comparison of its peak height with that of separately injected standard solutions. Analysis of UMP and CMP was performed on a Partisil-SAX column using isocratic elution [17] with 50 mM formic acid and 5 mM KH_2PO_4 (pH 4.1) at 1.5 ml/min.

Bone marrow cells were collected from both femurs by rinsing them with PBS, pH 7.4. The cells were washed, counted and extracted with 5% TCA. After removal of denatured protein the supernatant was neutralized with alamine–Freon as described above. Ribonucleoside triphosphates (UTP, CTP, ATP and GTP) were determined using a Partisil-10-SAX column using isocratic elution with 250 mM KH_2PO_4, 500 mM NaCl, pH 4.0.

Statistical evaluation was performed by using Student's t-test for paired and unpaired samples.

Assay of DHO-DH. The activity of DHO-DH was determined in mitochondria of Colon 26 and Colon 38 and in crude extracts of bone marrow cells. Tumors were removed from mice immediately after cervical dislocation, and put into ice-cold PBS. Mitochondria were isolated by differential centrifugation. Tumors depleted from visible necrotic parts were homogenized in a Potter–Elvejhem apparatus, the suspension was centrifuged at 600 *g* for 10 min at 4° and the 600 *g* supernatant subsequently at 7000 *g* for 20 min at 4°. The pellets containing the mitochondria were used for measurement of DHO-DH activity, and were used either directly or frozen at −20° until analysis. The enzyme remained stable for several months under these conditions. The inhibition of DHO-DH in mitochondrial pellets obtained from mice treated with DUP-785 was also retained during storage. Bone marrow cells were isolated as described above; the cell pellets were stored at −70°.

DHO-DH in colon tumors was assayed in mitochondria suspended in 0.1 M Tris–HCl, pH 8.0. The suspension was sonicated (2 × 5 sec with an interval of 10 sec at 4°, MSE sonicator) to get a homogenous solution. The assay mixture (final volume 500 μl) contained 20–500 μg protein, and the reaction was initiated by addition of L-DHO (final concentration 130 μM). The assay was performed at 37° and was terminated after 10–30 min. After precipitation of

Table 1. Antitumor effect of DUP-785 on Colon 26 and Colon 38

Parameter	Colon 26 (in Balb-c)	Colon 38 (in C57Bl/6)
Doubling time (days)	2.7	5.1
GDF	0.0; 0.14; 0.29	0.84; 1.32
% T/C (day)	86 (3); 98 (4); 86 (4)	40 (15); 48 (17)
ILS (%)	138; 109; 105	NA

DUP-785 was injected i.p. at 50 mg/kg at day 0, 4, 8 and 12. % T/C, volume of treated tumors divided by that of control tumors × 100%; within brackets the days at which optimal T/C was achieved. ILS, increase in life span; NA, not applicable for Colon 38 since these mice were killed when tumor volume exceeded 2000 mm^3. A GDF <1 is considered to represent resistance, >1 moderate sensitivity. A % T/C <50% also represents moderate sensitivity. Values of Colon 26 are of three different experiments, those of Colon 38 of two.

Table 2. Effect of DUP-785 on plasma uridine levels of BALB-C mice treated with DUP-785

Time (hr)	Non-treated	DUP-785 50 mg/kg	DUP-785 100 mg/kg
0	100	100	100
1	102 ± 33	63 ± 3*	64 ± 10*
2	90 ± 11	37 ± 7**	44 ± 8**
4	116 ± 16	47 ± 4**	35 ± 6***
24	98 ± 22	57 ± 10**	37 ± 4***

Blood samples were obtained from the same mice at the indicated time points. For each mouse the 0 hr sample was set at 100% and the other samples were calculated relative to this value. Mean relative values (±SE) of 4–8 mice are given. The plasma concentration of non-treated mice was 8.9 ± 1.2 μM (mean ± SE) of 12 mice. Differences were evaluated relative to the value at 0 hr with Student's t-test for paired samples. ***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$.

the denatured protein and neutralization with alamine–Freon, the amount of the product orotic acid, was determined using an automated HPLC assay using a Partisil-SAX column as described [21].

The activity of DHO-DH in bone marrow cells was determined after suspension of the bone marrow cells in 0.1 M Tris–HCl, pH 8.0 and sonication (2 × 5 sec with an interval of 10 sec at 4°). The assay mixture (500 μl) contained 0.5–10 × 10^6 cells and 130 μM L-DHO.

RESULTS

Antitumor effect of DUP-785

The antitumor effect of DUP-785 was evaluated by calculation of the GDF and the T/C (Table 1). At this schedule DUP-785 showed no activity against Colon 26 and a moderate activity against Colon 38. Toxicity of DUP-785 at this schedule was minor, weight loss was <5%.

Effect of DUP-785 on nucleoside and nucleotide pools

Balb-c mice were treated with single i.p. injections of DUP-785. One hundred mg/kg was studied since this was 80% of the maximum tolerated dose at single administration for these mice. No variation in uridine levels in non-treated mice was observed. At both 50 and 100 mg/kg a decrease in plasma uridine concentrations was observed within 1 hr (Table 2). The extent of the decrease was comparable for both doses but after 24 hr a small recovery was observed at 50 mg/kg while at 100 mg/kg uridine levels were still depressed. All other antipyrimidine effects were studied at a dose of 50 mg/kg since this dose could be used for therapeutic purposes, while repeated administration of 100 mg/kg would be too toxic for the mice.

Uridine pools were studied in both colon tumors and livers of the same animals. For Colon 38, care was taken that visible necrotic sections were separated from viable tissue. Colon 26 did not show necrosis. At times analysis of uridine was disturbed by interfering peaks in the chromatogram. Adjusting the pH and/or concentration of methanol improved the separation. In a previous paper [17] we demonstrated that our methods resulted in a reliable estimate of uridine tissue levels.

The basal levels of uridine differed markedly between the tumors and livers. In the same mouse strain uridine concentrations in livers were several times higher than in the tumors. In livers of Balb-c mice (bearing Colon 26) the uridine concentration was higher than in those of C57Bl mice (bearing Colon 38), while in tumor Colon 38 uridine concentrations were 10-fold higher than in tumor Colon 26. In a few tumors of non-treated mice uridine was measured both in necrotic and viable tissue. In necrotic tissue the uridine concentration was below 38 pmol/mg wet weight, which is lower than in viable tissue (Fig. 1). DUP-785 caused a sharp decrease (90%) of uridine concentrations in both Colon 38 and 26; which was followed in Colon 26 by a rapid recovery, in contrast to Colon 38 (Fig. 2). The effects on uridine pools observed in livers of these mice were less pronounced (Table 3).

Since in cell lines only pyrimidine nucleotide pools were affected by DUP-785, we limited the *in vivo* studies to these nucleotides. Total pyrimidine nucleotide pools were studied since during preparation of tissues, degradation of pyrimidine nucleoside polyphosphates to the monophosphate was observed but not to nucleosides and bases [17]. Furthermore, *in vitro* studies with DUP-785 and other pyrimidine *de novo* inhibitors [13, 22] had demonstrated that pyrimidine mono-, di- and triphosphates decreased to the same extent. UΣP and CΣP pools were comparable in Colon 26 and 38, UΣP pools being two-fold those of CΣP (Fig. 1). In Colon 26 administration of DUP-785 caused an initial increase

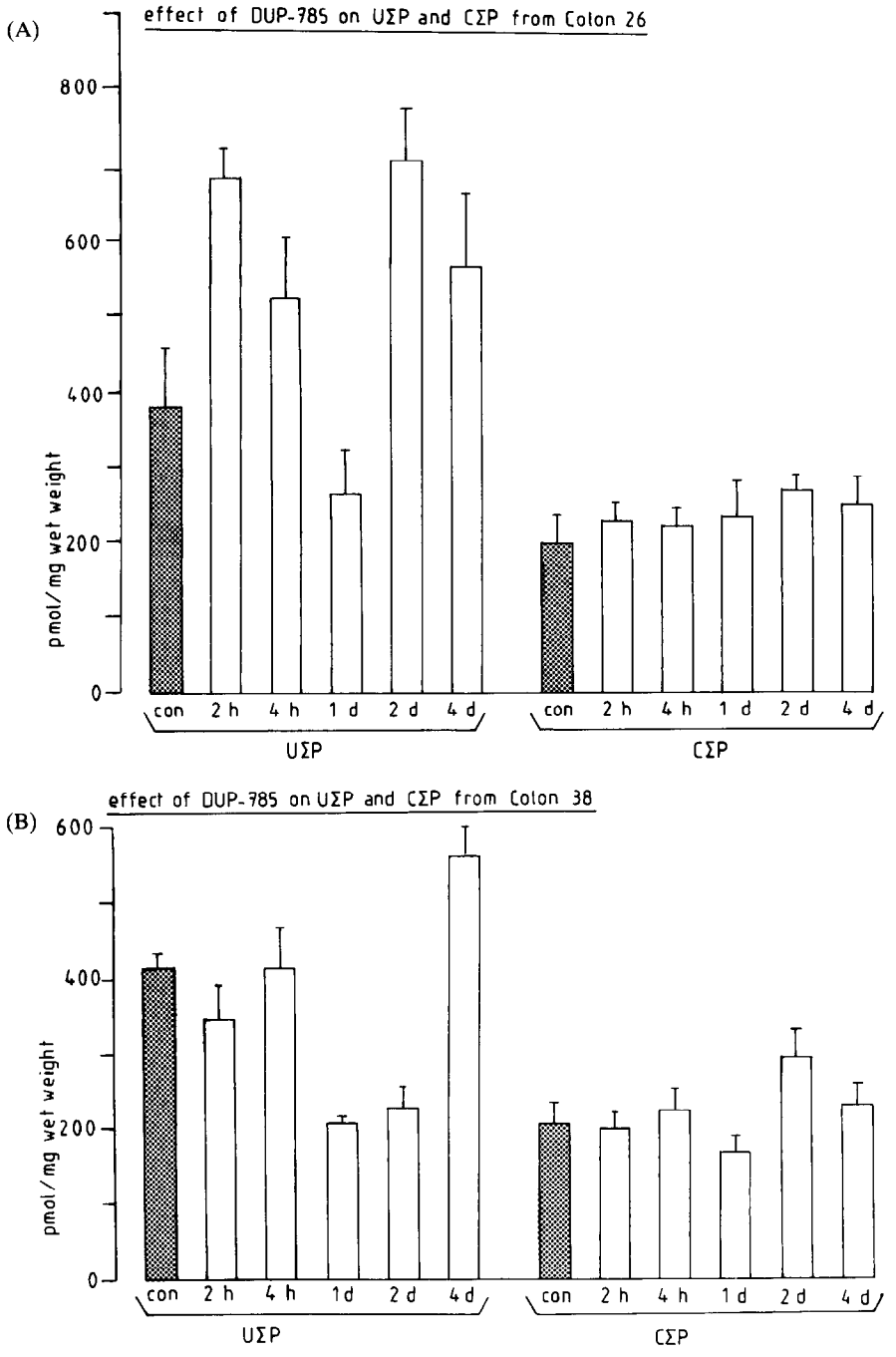

Fig. 1. Effect of DUP-785 at 50 mg/kg on the concentrations of UΣP and CΣP in Colon 26 (A) and Colon 38 (B). Bars represent means ± SE of 5–11 and 3–15 tumors, respectively. The Colon 26 control UΣP level was significantly different from levels at 2 hr and 2 days ($0.001 < P < 0.01$); the level of 1 day was significantly different from those at 2 hr and 2 days ($P < 0.001$) and at 4 hr and 4 days ($0.02 < P < 0.05$). The Colon 38 control UΣP level was significantly different from levels at 1 and 2 days ($P < 0.001$) and 4 days ($0.001 < P < 0.01$); the level of 4 days was significantly different from those at 1 and 2 days ($P < 0.001$).

in UΣP after 2 and 4 hr followed by a small decrease after 1 day. This decrease was followed by a rebound to 175% of the control levels. DUP-785 failed to reduce CΣP levels, while a slight increase of 30% was observed after 2 days. In Colon 38 DUP-785 did not affect the level of UΣP after 2 and 4 hr, but after 1 and 2 days a significant decrease of about 40% was found. After 4 days a small rebound in UΣP was observed, which was significant compared to the levels of 1 and 2 days. CΣP was somewhat decreased after 1 day followed by an increase after 2 days. The CΣP levels after 2 days were significantly different from those of 1 day after DUP-785 treatment.

Pyrimidine nucleotide pools in livers of Balb-c and

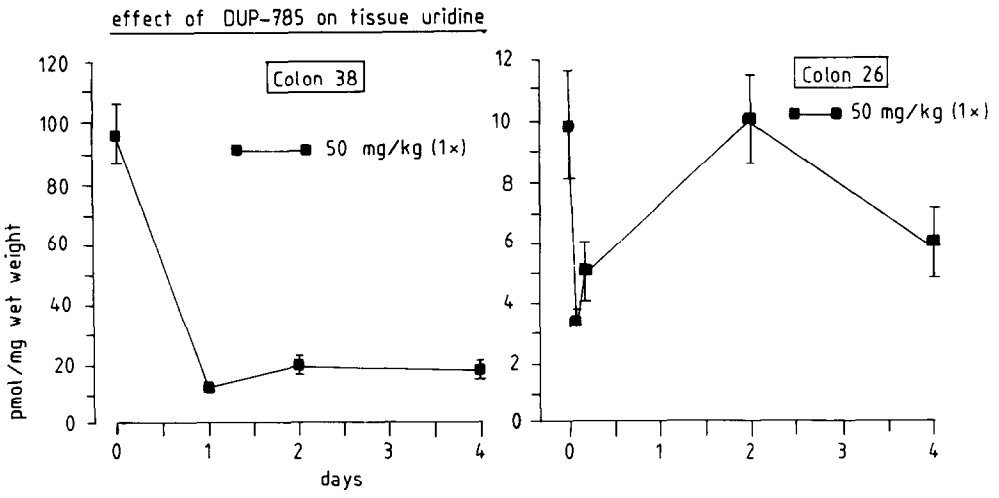

Fig. 2. Effect of i.p. DUP-785 at 50 mg/kg on the levels of uridine in Colon 38 (grown in C57Bl/6 mice) and Colon 26 (grown in Balb-c mice). Values are means ± SE of 4–13 tumors (Colon 38) and 5–11 tumors (Colon 26). Levels of 1, 2 and 4 days of Colon 38 were significantly different ($P < 0.001$) from control levels.

Table 3. Effect of DUP-785 (50 mg/kg) on uridine levels in livers of Balb-c and C57Bl/6 mice

Time (days)	C57Bl/6	Balb-c
0	346 ± 115 (5)	880 ± 24 (3)
1	429 ± 87 (3)	563 ± 102 (5)*
2	545 ± 54 (8)	723 ± 135 (4)
4	516 ± 195 (3)	555 ± 70 (4)*

Values (in pmol/mg wet wt) are means ± SE for the number of livers indicated within parentheses. Significantly different from control levels; * $0.01 < P < 0.05$.

C57Bl mice were comparable, but the mouse strains were different in their response to treatment with DUP-785 (Fig. 3). In livers of Balb-c mice a small decrease was followed by an increase in both CΣP and UΣP, in livers of C57Bl/6 mice a decrease in UΣP was found after 2 days.

The different effects of DUP-785 on CΣP and UΣP pools are reflected more clearly by calculation of the ratio UΣP/CΣP (Table 4). The ratio was higher in livers. The most immediate and pronounced effect of DUP-785 was observed in Colon 38. A short-lasting effect was found in Colon 26. In livers of Balb-c mice the effect of DUP-785 on the ratio was minor, but in those of C57Bl a marked effect was observed.

The number of viable (>95%) bone marrow cells (based on Trypan blue exclusion) was not decreased after treatment with DUP-785. However, DUP-785 not only decreased UTP but also ATP and GTP. This decrease in purine nucleotides indicated that the condition of bone marrow cells is worse than based on Trypan blue exclusion. Recovery of purine nucleotides paralleled that of UTP (Fig. 4).

Effect of DUP-785 on the activity of DHO-DH

The activity of DHO-DH and the inhibition by DUP-785 was determined in Colon 26 and 38 and in bone marrow cells of non-treated Balb-c and C57Bl mice. The basal activity of DHO-DH in mitochondria of Colon 26 was three-fold higher than in Colon 38 (Fig. 5); the inhibition by 1.3 μM DUP-785 was 94 and 82%, respectively. So, residual activity in the presence of DUP-785 was comparable in both tumors. The activity of DHO-DH in bone marrow cells of Balb-c and C57Bl mice was comparable (0.98 ± 0.16 and 1.25 ± 0.15 nmol orotic acid/hr per 10^6 cells, respectively; means ± SE of four mice), inhibition by 1.3 μM DUP-785 was more than 90%.

Retention of DHO-DH was measured in tumors and bone marrow cells, since in these tissues the most pronounced effect on pyrimidine nucleotide pools was observed. In addition clinical toxicity consisted, among others, of bone marrow suppression but not of liver toxicity.

In both colon tumors treatment with DUP-785 almost completely inhibited DHO-DH at 4 hr after administration (Fig. 5). Although the percentage of inhibition in Colon 26 was higher, the absolute levels of DHO-DH were comparable in both tumors. In both tumors partial recovery of DHO-DH was observed as early as 1 day after administration of the drug. In Colon 38 DHO-DH levels were back to control levels after 4 days. Since in Colon 26 no complete recovery was found, levels were also measured after 7 days. These appeared to be comparable to those in Colon 38, in which even an increase compared to basal levels was observed. In bone marrow cells of the same Balb-c and C57Bl mice the same pattern of DHO-DH was found, so only the curve for C57Bl mice is shown (Fig. 6). After 4 hr DHO-DH was almost completely inhibited and recovered slightly at 1 and 2 days. After 4 and 7 days DHO-DH continued to recover but did not reach control levels.

DISCUSSION

In this paper we provide evidence that treatment with DUP-785 produces clear antipyrimidine effects in tumors and bone marrow, which is manifested as

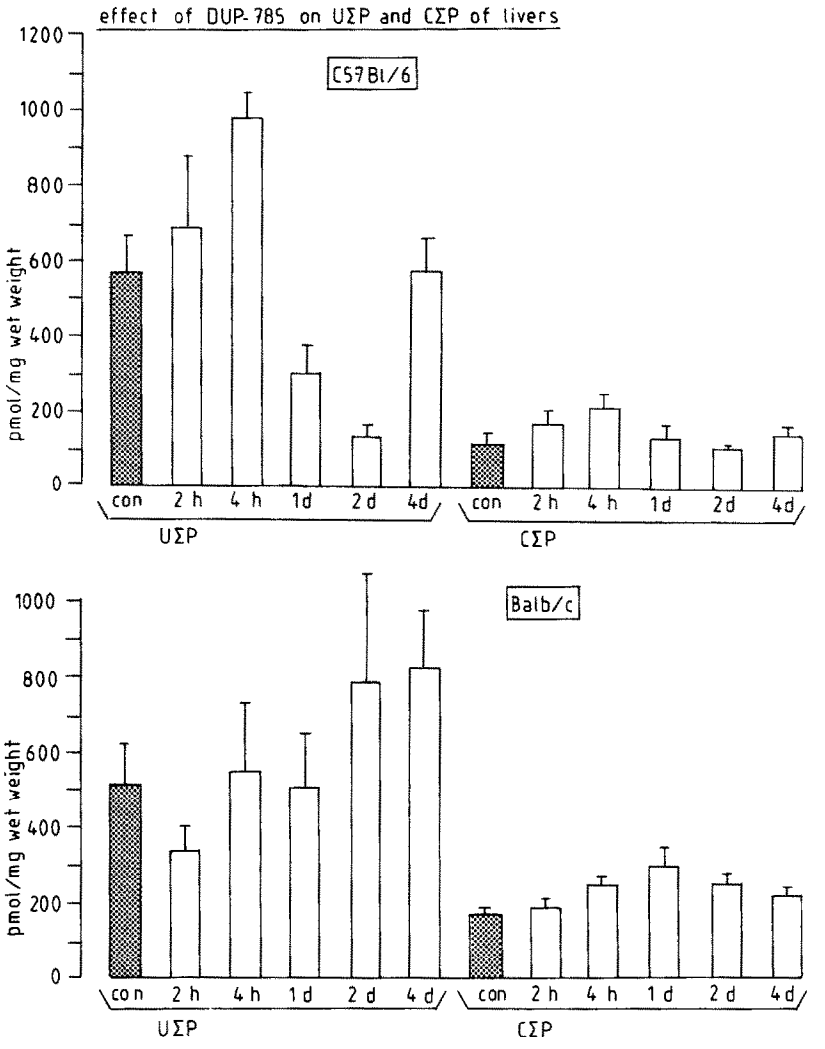

Fig. 3. Effect of DUP-785 at 50 mg/kg on the concentrations of UΣP and CΣP in livers of C57Bl/6 (bearing Colon 38) and Balb-c (bearing Colon 26) mice. Bars represent means ± SE of 3–8 livers. For C57Bl/6 the control UΣP level was significantly different from that at 2 days ($0.001 < P < 0.01$); the UΣP level at 4 days was significantly different from those at 4 hr ($0.001 < P < 0.01$) and 2 days ($0.01 < P < 0.02$). The 4 hr CΣP level was significantly different from the control ($0.02 < P < 0.05$) and that at 2 days ($0.001 < P < 0.01$). For Balb-c the UΣP levels at 2 hr and 4 days were significantly different ($0.02 < P < 0.05$) from each other; the CΣP control level was significantly different from those at 4 hr ($0.001 < P < 0.01$) and 1, 2 and 4 days ($0.02 < P < 0.05$).

Table 4. Effect of DUP-785 (50 mg/kg) on the UΣP/CΣP ratios in the colon tumors Colon 26 and Colon 38 and the livers from the host mice, Balb-c and C57Bl/6, respectively

	Tumors		Livers	
Time	Colon 26	Colon 38	Balb-c	C57Bl/6
0 hr	2.21 ± 0.38	2.19 ± 0.71	3.05 ± 0.99	3.64 ± 1.47
2 hr	2.98 ± 0.13	1.78 ± 0.20	1.83 ± 0.18	3.86 ± 0.64
4 hr	2.59 ± 0.61	1.78 ± 0.15	2.11 ± 0.55	4.80 ± 0.86
1 day	1.12 ± 0.47*	1.23 ± 0.16**	2.02 ± 0.92	1.90 ± 0.29
2 days	2.77 ± 0.12	0.90 ± 0.09***	3.05 ± 0.52	1.40 ± 0.32*
4 days	2.22 ± 0.22	2.40 ± 0.32	3.85 ± 0.83	3.60 ± 0.51

Ratios were calculated from the separate UΣP and CΣP values from the same tissue sample. Values represent means ± SE of 3–13 mice. Significantly different from levels at 0 hr: *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

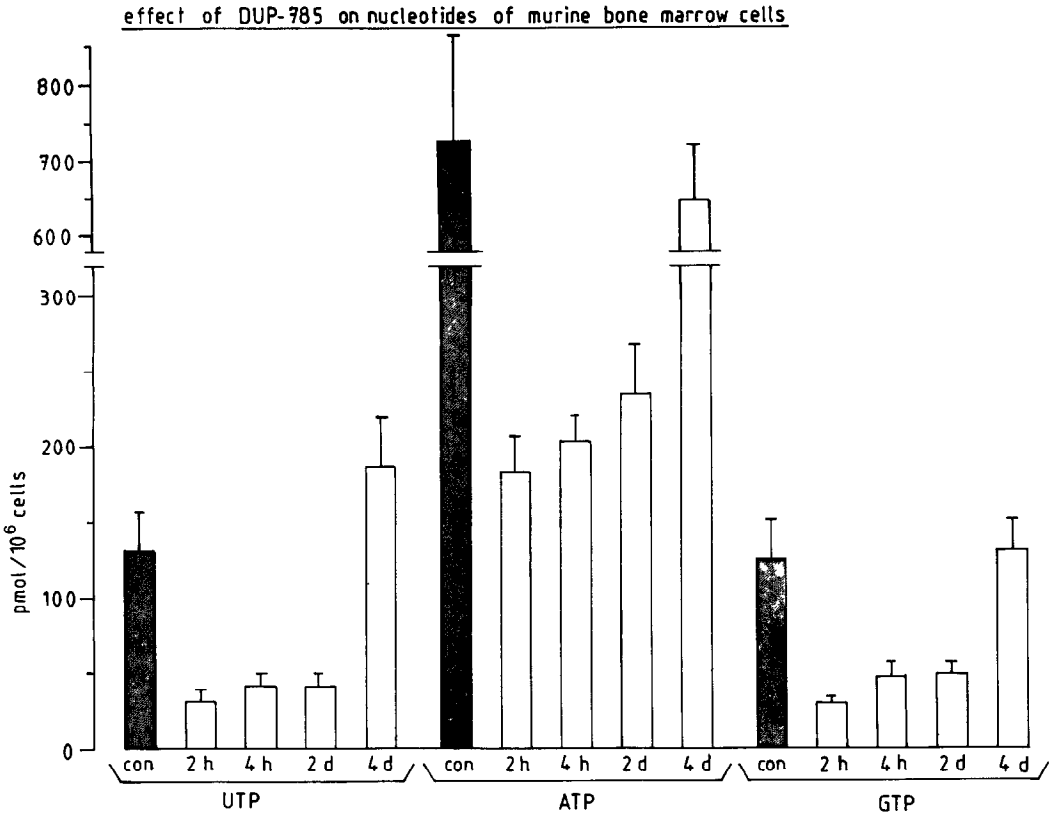

Fig. 4. Effect of DUP-785 at 50 mg/kg on the concentrations of UTP, ATP and GTP in bone marrow cells isolated from Balb-c mice. Values represent means ±SE of four mice. All values (except those of 4 days) were significantly different ($0.001 < P < 0.01$) from control values. From each mouse bone marrow cells from two femurs were pooled.

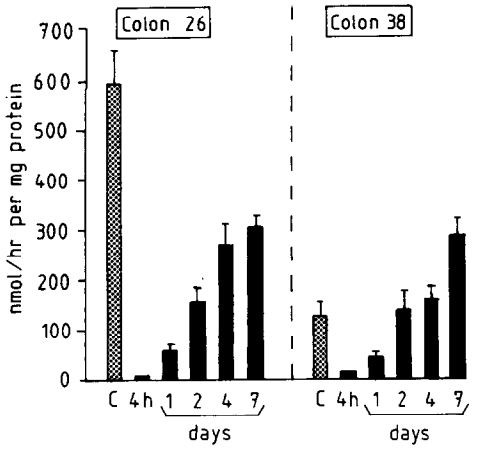

Fig. 5. Effect of DUP-785 at 50 mg/kg on the activity of DHO-DH in mitochondria isolated from Colon 26 and Colon 38. Bars represent means ±SE of 4–8 tumors. The decrease in activity after 4 hr and 1 day was significantly different ($P < 0.01$) from control values in both tumors, other values of Colon 26 and the rebound of Colon 38 were significantly different from the control at the level $0.001 < P < 0.01$.

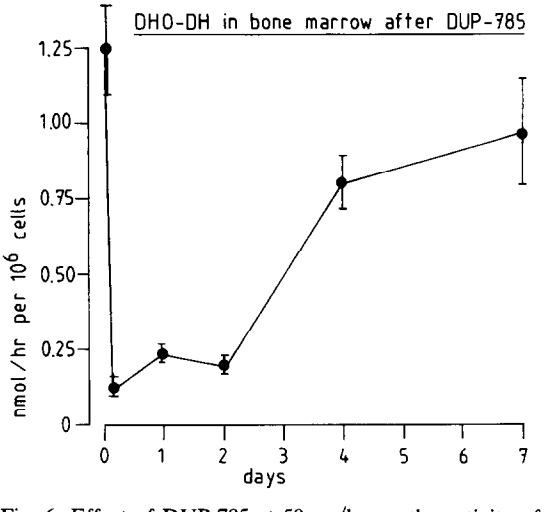

Fig. 6. Effect of DUP-785 at 50 mg/kg on the activity of DHO-DH in bone marrow cells isolated from Balb-c mice. Values are from 3–5 mice and are means ± SE. The activities at 4 hr, 1 and 2 days were significantly different ($P < 0.001$) from control levels.

DHO-DH inhibition and the subsequent decrease in pyrimidine nucleotides. The latter seems to be, at least partly, related to its antitumor effect.

Earlier DUP-785 has been shown to cause marked reduction of pyrimidine nucleotides in both leukemic and colon cancer cell lines [11, 13]. Retention of this depletion and of inhibition of DHO-DH [12, 13] correlated wll with the extent and duration of growth-inhibition. Although the *in vitro* depletion of pyrimidine nucleotides was more than 90%, this was not observed *in vivo* for solid tumors. The highest decrease was observed in Colon 38, but was not more than 50%. In both tumors a rather limited rebound was found. A rebound was also observed in the activity of DHO-DH in Colon 38 but not in Colon 26.

The depletion of UTP in bone marrow cells accompanied by a decrease in ATP and GTP indicates a pronounced effect of DUP-785 on cellular energy metabolism, which might be related to the mitochondrial location of DHO-DH and the relation with the electron transport chain. It might be possible that these cells still have the capability to recover, since no depletion of bone marrow cells was observed and all nucleotides reached normal levels after 4 days.

Administration of DUP-785 to mice resulted in a rapid inhibition of DHO-DH in both tumors and bone marrow. The DHO-DH inhibition in bone marrow cells is comparable to that of DHO-DH in lymphocytes of patients treated with DUP-785 [14] and to *in vitro* inhibition of DHO-DH in L1210 cells [11]. Furthermore in bone marrow cells and L1210 this inhibition is accompanied by an immediate depletion of pyrimidine nucleotides [12, 13], while the pattern of recovery of DHO-DH is almost identical to that of pyrimidine nucleotides. In contrast, in both tumors DHO-DH is also almost completely inhibited, but this is not accompanied by an immediate depletion in pyrimidine nucleotides. In Colon 26 treatment with DUP-785 even resulted in an increase of uridine nucleotides, while uridine in tissue and plasma decreased. Probably the tissue and plasma uridine pool is used very efficiently to replenish the uridine nucleotide pools of the Colon 26 tumor; this tumor has a very high uridine kinase compared to e.g. Colon 38 [23]. This lower capacity of Colon 38 to salvage uridine may explain why no increase in uridine nucleotides is found after 2 and 4 hr, and a small decrease after 1 and 2 days. The stronger antipyrimidine effect in Colon 38 is expressed more clearly by the imbalance in pyrimidine nucleotide pools, expressed as the UΣP/CΣP ratio. *In vitro* the lowest UTP/CTP ratio corresponded to the largest growth inhibition [13]. We found the lowest UΣP/CΣP ratio in Colon 38, for which a better antitumor effect was observed.

In livers no effect on the uridine pool was observed in both mouse strains, in contrast to plasma. Besides a high rate of pyrimidine *de novo* synthesis livers also have a high capacity to synthesize uridine nucleotides via the salvage pathway [2, 3]. Combined with the concentrative uptake of uridine [24, 25] in tissues from plasma, this might explain why uridine is depleted in plasma but not in liver. The source of uridine may consist of diet and/or breakdown of nucleic acids.

In vivo biochemical effects of DUP-785 are unlike those observed with PALA [9, 10, 26, 27] or pyrazofurin [28], other inhibitors of the pyrimidine *de novo* pathway (Fig. 7). The extent of depletion of pyrimidine nucleotides as we now observed with DUP-785 was less, while no rebound was observed with PALA. However, in most of these studies the nucleotide pools were only measured at 24 hr. We observed in B16 tumors that both UTP and CTP remained depleted for 5 days [29]. Furthermore, in Lewis lung carcinoma one injection of PALA resulted in inhibition of ATC which was retained for 6 days [26]. Retention of DHO-DH inhibition and the effects on UΣP lasted only a few days in our murine tumors. It should be noted that although the extent of inhibition in the Colon 26 was higher than in Colon 38, that the absolute DHO-DH activity in Colon 26 was never below that in Colon 38, while the increase in Colon 26 was faster. So, it might be that *in vivo* the residual absolute activity of DHO-DH is important.

Despite the limited antipyrimidine effects, as compared to PALA or pyrazofurin, DUP-785 shows a broad antitumor activity against both experimental tumors and human xenografts [30]. In this respect it is of interest to recall that the role of DHO-DH in cellular function is unclear. A number of observations should be mentioned. During hepatocarcinogenesis DHO-DH activity appeared to be decreased [31], while cells with defective DHO-DH did not require exogenous uridine for growth [32]. Depending on growth conditions cytidine even potentiated the effect of another inhibitor of DHO-DH, aza-dihydroorotic acid [33]. This particular enzyme in the pyrimidine pathway is unique because of its location in the mithochondrion [1–3], while it is coupled to the electron transport chain [34]. Thus, the enzyme has been demonstrated to drop under hypoxic conditions [35], while hypoxic conditions also appeared to be synergistic with another inhibitor of DHO-DH [35]. It is not yet clear whether hypoxia might be an important factor in the *in vivo* activity of DUP-785. DUP-785 is rapidly taken up by tissues [36], and very likely also in the mitochondrion, considering the inhibition of DHO-DH. It has been demonstrated that after 24 hr tissue levels are about 10 μg/g tumor tissue [36], a level high enough to inhibit DHO-DH *in vitro* [12] and *in vivo* (Figs 5 and 6). However, considering the data in this report inhibition of DHO-DH may be a factor leading to antitumor activity, but the capacity of a tumor to replenish pyrimidine nucleotide pools may be a limitation. Nucleoside pools vary considerably in various tissues [17, 25], which may determine the antitumor effect. The rather low uridine in bone marrow [37] provides a small source for salvage of pyrimidine nucleotides, which might explain the bone marrow depression observed in patients [38]. For another antimetabolite, 5-fluorouracil, we could limit bone marrow depression in mice and patients [16, 39] using biochemical modulation with uridine. Biochemical modulation with e.g. uridine may be a possibility for selective protection of bone marrow depression but not affect the antitumor activity of DUP-785.

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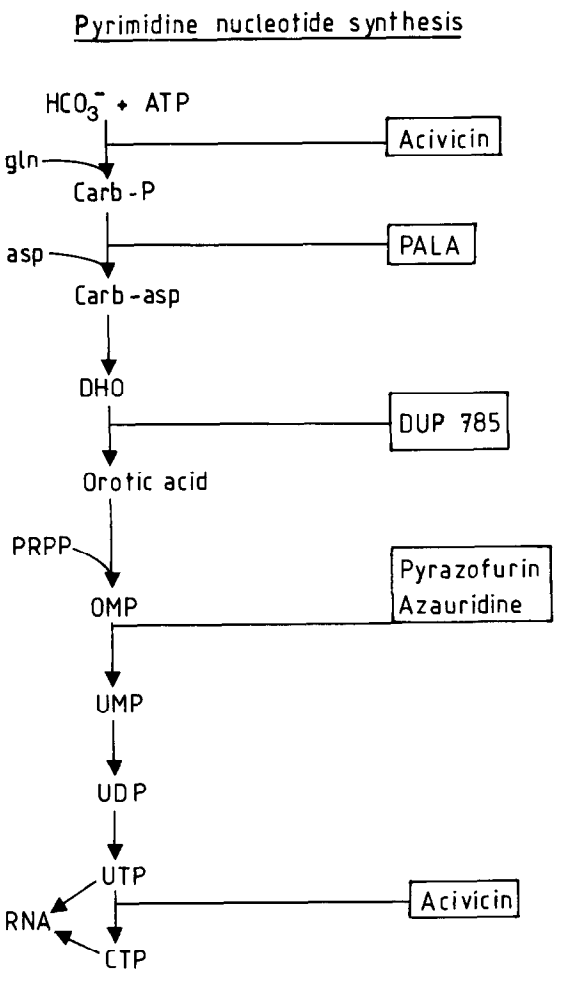

Fig. 7. Inhibition of the pyrimidine *de novo* enzyme, DHO-DH, by DUP-785, in comparison to other pyrimidine *de novo* enzyme inhibitors, acivicin, PALA, pyrazofurin and azauridine. Carb-P, carbamyl-phosphate; Carb-asp, carbamyl-aspartate; gln, glutamine; asp, aspartate; PRPP, 5-phosphoribosyl-1-pyrophosphate.

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