14. Pearce S, Dowsett M, Jeffcoate SL. Three methods compared for estimating the fraction of testosterone and estradiol not bound to sex-hormone-binding globulin. *Clin Chem* 1989, **35**, 632–635
15. O'Connor S, Baker HWG, Dulmanis A, Hudson B. The measurement of sex steroid binding globulin by differential ammonium sulphate precipitation. *J Steroid Biochem* 1973, **4**, 331–339.
16. Moore JW, Key TJA, Bulbrook RD, *et al.* Sex hormone binding globulin concentrations in a population of normal women who had never used exogenous hormones. *Br J Cancer* 1987, **56**, 661–666.

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# Thymidylate Synthase from Untreated Human Colorectal Cancer and Colonic Mucosa: Enzyme Activity and Inhibition by 5-fluoro-2′-deoxyuridine-5′-monophosphate

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**Inhibition of thymidylate synthase (TS) by the 5-fluorouracil (5-FU) metabolite FdUMP is considered to be the main mechanism of action of 5-FU. TS from colorectal tumours and normal colon mucosa from 10 untreated patients was studied. There was a large variation in the activity of tumour TS both at 1 and 10 μmol/l of its substrate dUMP; in normal mucosa this variation was less. Inhibition by 10 nmol/l FdUMP in tumours varied from 80 to 90% at 1 μmol/l dUMP; in normal mucosa, inhibition varied from 10 to 80%. The number of FdUMP binding sites ranged from 0.1 to 1 in tumours but such binding sites were not detectable in normal mucosa. The ratio between TS activity and FdUMP binding sites varied considerably in tumours but not in normal mucosa. The deviations from normal kinetics may represent a mutant TS form. Alterations in TS may partly account for differences in response to 5-FU.**
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## INTRODUCTION

THYMIDYLATE SYNTHASE (TS) is a key enzyme in the *de novo* synthesis of thymidine nucleotides, for which deoxyuridine 5′-monophosphate (dUMP) is the substrate and 5,10-methylenetetrahydrofolate ($CH_2$-THF) the methyl donor. The $K_m$ for the substrate is about 1–5 μmol/l [1–4], while the $K_m$ for $CH_2$-THF varies between 10 and 50 μmol/l. Inhibition of TS by the metabolite 5-fluoro-2′-deoxy-UMP (FdUMP) is one of the main mechanisms of action of 5-fluorouracil (5-FU). 5-FU is one of the few drugs useful in colorectal cancer [4]. Leucovorin, a precursor for $CH_2$-THF, potentiates the effect of 5-FU in patients [5], in mice [6–8] and *in vitro* [8]. Inhibition of TS by FdUMP is probably of crucial importance in the action of 5-FU in patients [8–10].

TS is inhibited by the formation of a covalent ternary complex between the enzyme, FdUMP and $CH_2$-THF [2–4]. This complex is rapidly formed and the rate of dissociation may determine the efficacy of 5-FU. TS can also be inhibited by an unstable binary complex between FdUMP and TS [4, 11, 12]. In cell and tissue extracts FdUMP is a potent competitive inhibitor of TS ($K_i$ about 1 nmol/l). Retention of inhibition is mainly determined by the stabilisation of the ternary complex by $CH_2$-THF or one of its polyglutamates [13, 14]. In addition the concentrations of FdUMP and dUMP will influence the extent of inhibition. *In vitro*, resistance to 5-FU or 5-fluoro-2′-deoxyuridine (FUdR) has been related to altered kinetics of TS for dUMP and FdUMP binding [2, 4, 15], disturbed folate pools [14] and the level of enzyme before treatment [15]. Gene amplification of TS has been demonstrated for FUdR-resistant cell lines [16]. Evidence for gene amplication has also been obtained in a patient with colon cancer who developed resistance against 5-FU [17], while in breast cancer patients binding of FdUMP and the effect of $CH_2$-THF decreased during development of resistance [9].

These aberrations in kinetic properties may affect the extent and duration of inhibition of TS by FdUMP, perhaps even precluding synergism between LV and 5-FU. For instance, one form of TS in a cell line was resistant to inhibition by FdUMP [18]. Therefore we have measured the activity of TS, inhibition by FdUMP and the binding of FdUMP to TS in biopsy specimens of colorectal tumours from previously untreated patients. To establish whether gastrointestinal toxicity may be attributed to enzyme inhibition, we also studied adjacent normal mucosa. Part of the data have been reported in preliminary form [19].

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## MATERIALS AND METHODS

### *Chemicals*

Tetrahydrofolate (THF), collagenase and DNAse were purchased from Sigma. The synthesis of $CH_2$-THF from THF has been described [20]. [5-$^3$H]-dUMP and [6-$^3$H]-deoxyuridine were obtained from Amersham International. [6-$^3$H]-FdUMP was obtained from Moravek Biochemicals, Brea, California. All other chemicals were of standard analytical quality.

### *Enzyme preparation*

Biopsy specimens of primary colorectal tumours and adjacent healthy mucosa (free from underlying muscle) from untreated patients were obtained as soon as possible after surgical removal, and immediately frozen and stored in liquid nitrogen. Care was taken that visible necrotic regions were not frozen. Under these conditions the enzyme was stable for at least 3 years and biochemical properties of stored tissues were similar to those of the same tissues assayed immediately. Frozen tissues were pulverised in a microdismembrator [21]. The powder was weighed and suspended in assay buffer (50 mmol/l Tris-HCl, 1 mmol/l EDTA, 10 mmol/l dithiothreithrol, pH 7.4) at 1 g tissue per 3–4 ml buffer. The suspension was centrifuged at 2500 *g* (5 min, 4°C) and the supernatant was centrifuged at 11 000 *g* (10 min, 4°C). The supernatant was immediately used for measurement of TS.

### *Assays of TS*

All assays were done at 37°C in a shaking water-bath. The TS catalytic assay and the FdUMP binding assay were done with the 11 000 *g* supernatants. It is unlikely that intrinsic cytosolic dUMP levels interfere with the assay, since the homogenate is diluted several times during preparation and subsequently in the assays. The catalytic activity of TS was measured with a tritium release assay at 1 and 10 μmol/l [5-$^3$H]-dUMP [20]. The reaction mixture (total volume 50 μl) contained 50–500 μg protein (equivalent to 2–25 μl of 11 000 *g* supernatant). To inhibit breakdown of nucleotides we also added 100 mmol/l NaF and 15 mmol/l cytidine monophosphate (CMP). The incubation time was usually 30 and/or 60 min; the reaction was linear with time (up to 60 min) and protein concentration. The assay was terminated by the addition of 50 μl 35% trichloroacetic acid (TCA) and unreacted [5-$^3$H]-dUMP was bound by the addition of 250 μl 10% activated charcoal. FdUMP binding was assayed essentially as described [22]. 11 000 *g* supernatant (total volume of assay 250 μl; 100–1000 μg soluble protein, equivalent to about 10–100 μl 11 000 *g* supernatant) was incubated with [6-$^3$H]-FdUMP and $CH_2$-HF (30 nmol/l and 350 μmol/l final concentrations, respectively) for 1 h. We also added 100 mmol/l NaF and 15 mmol/l CMP. The incubation was terminated by addition of 500 μl 10% activated charcoal in 0.2 mol/l HCl to bind free [$^3$H]-FdUMP; the suspension was centrifuged and radioactivity in the supernatant was counted. For both the catalytic and FdUMP binding assays blanks were about 1000 dpm; only reactions which gave at least 1.5–2 times higher dpm were evaluated.

From several patients enough tumour material could be obtained to isolate intact tumour cells. After surgical removal the tumour was immersed in DMEM containing 20% fetal bovine serum buffered with 20 mmol/l HEPES in the presence of penicillin and streptomycin. Viable-appearing regions of the tumours were removed, cut into small pieces and incubated (1 g per 5 ml) with collagenase (1.5 mg/ml) and DNAse (200 μg/ml) for 45 min at 37°C. After the incubation the tissues were filtered twice over a stainless steel screen (250 mesh, then 40 mesh) and the filtered cells were precipitated by centrifugation (2 min at 4°C at 400 *g*), suspended in DMEM and counted in a haemacytometer. The pieces of mucosal cells were washed several times with phosphate-buffered saline pH 7.4 until the supernatant was clear. Colonocytes (colon mucosa cells) were prepared by the chelation–elution method [23]. These procedures gave a yield of about $27.10^6$ tumour cells and $7.10^6$ colonocytes per g tissue. The cell suspensions were incubated in 100 μl ($0.2 \times 10^6$ cells) in flat-bottom 96-well plates in the absence and presence of 1 and 10 μmol/l 5-FU for 1 h; [6-$^3$H]-deoxyuridine (final concentration 0.1 μmol/l; 0.7 TBq/μmol) was added. After incubation for 2 h the high molecular material was precipitated on glass-fibre filters in a cell-harvester and radioactivity was estimated after solubilisation [24]. The conditions for the [6-$^3$H]-deoxyuridine test were optimised in two human colorectal cell lines (WiDr and Intestine 407, data not shown) that had been previously characterised [20, 33]. The difference in [6-$^3$H]-deoxyuridine incorporation between the cell lines was similar to that of the TS activity. In these cell lines, as well as in isolated tumour cells, [6-$^3$H]-deoxyuridine incorporation increased linearly with time.

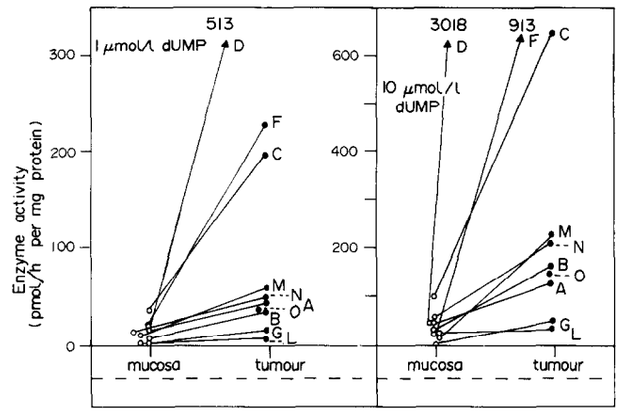


**Fig. 1. Activity of TS in biopsy specimen of human colon tumours (closed circles) and colonic mucosa (open circles). Lines connect samples from the same patient. Activity (both at 1 and 10 μmol/l dUMP) in tumours was significantly higher than in mucosa ($P < 0.001$, two-tailed $U$ test). Amount of soluble protein in both tumour and mucosa samples was about 33 mg/g wet weight.**

## RESULTS

The tumours were obtained from 10 patients with histologically proven colorectal cancer. The differentiation varied from poor to moderate. Dukes' classification varied from A to C. The median age of the patients was 56 (range 54–90). Biochemical variables were not correlated with clinical response because most patients were not treated with chemotherapy subsequently.

The activity of TS and its inhibition by FdUMP were assayed at 1 and 10 μmol/l dUMP to detect variations in kinetics [20]. Saturating $CH_2$-THF (as a racemic mixture) concentrations (350 μmol/l) were used. In tumours, phosphatase activity is high [17, 25]. Addition of NaF and CMP to the TS assay to inhibit breakdown of nucleotides (dUMP and FdUMP) led to contradictory results; in most samples TS activity was somewhat higher, but in one sample the activity was even lower. All reported values contained NaF and CMP in the assay mixture. There was a large variation in the activity of TS in tumours, more so at 10 than at 1 μmol/l dUMP (Fig. 1). In mucosa the

*Table 1. Relation between TS activity of tumours and mucosa*

| Tumour | Ratio tumour/mucosa* 1 μmol/l dUMP | Ratio tumour/mucosa* 10 μmol/l dUMP | Ratio between 10 and 1 μmol/l dUMP† Tumour − FdUMP | Tumour + FdUMP | Mucosa − FdUMP | Mucosa + FdUMP |
|---|---|---|---|---|---|---|
| A | 2.5 | 2.9 | 3.1 | 11.3 | 2.7 | 6.8 |
| B | 4.1 | 3.8 | 3.1 | 10.0 | 2.7 | 9.6 |
| C | 5.0 | 6.1 | 3.2 | 8.4 | 2.6 | 8.0 |
| D | 27.9 | 28.5 | 5.9 | 6.8 | 5.8 | 11.8 |
| F | 12.0 | 15.8 | 4.0 | 9.0 | 3.1 | 4.2 |
| G | 5.2 | 7.0 | 3.6 | ND | 2.6 | ND |
| L | 1.1 | 1.0 | 2.8 | NA | 3.2 | NA |
| M | 8.2 | 9.7 | 3.8 | 23.6 | 3.2 | 4.4 |
| N | 3.3 | 3.7 | 4.3 | 7.8 | 3.8 | 3.0 |
| O | NA | NA | 4.1 | 6.4 | NA | NA |

*Ratio between 10 and 1 μmol/l dUMP was significantly higher in tumours than in normal mucosa ($P < 0.05$, one-tailed Mann–Whitney $U$ test).

†Ratios between 10 and 1 μmol/l in the absence of FdUMP were significantly lower than in presence of FdUMP ($P < 0.001$ in tumours and $P < 0.01$ in mucosa).

NA = not available; for patient O no mucosa sample was available, while for patient L not enough sample was available to measure inhibition of TS at 10 μmol/l dUMP in mucosa. ND = not detectable.

variation in the activity of TS, at both dUMP concentrations was lower. However, in all patients (except L) TS activity was higher in the tumour than in mucosa, varying between 1 and 28 fold. The ratio of TS activity between 10 and 1 μmol/l dUMP in tumours varied between 2.8 and 5.9, the highest ratio was found at the highest activity. In mucosa this ratio varied between 2.6 and 5.8 (Table 1).

Inhibition by FdUMP was measured by simultaneous addition of both dUMP and FdUMP. In tumours, inhibition by 10 nmol/l FdUMP varied between 40 and 90%, being higher at 1 than at 10 μmol/l (Fig. 2). In healthy mucosa of most patients, inhibition by FdUMP (at 10 μmol/l dUMP between 0 and 80%) was less than in tumours. The large variation in inhibition by FdUMP between tumours and mucosa was also expressed by the variation in the ratio of TS activity at 10 and 1 μmol/l FdUMP (Table 1).

FdUMP binding in tumour samples was measured in the

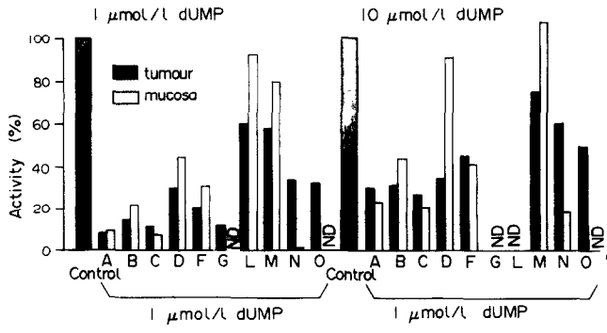


**Fig. 2. Inhibition of TS by 10 nmol/l FdUMP expressed as activity of TS in the presence of FdUMP compared with activity in absence of FdUMP. ND = not detectable in patient G or not done in patients and O since not enough material was available. Control bar represents TS activity in the absence of FdUMP.**

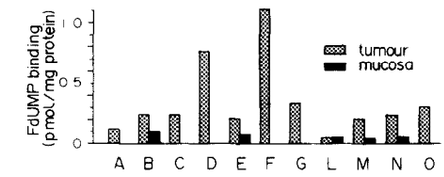


**Fig. 3. Binding of FdUMP to TS. Number of binding sites for FdUMP in tumours was significantly higher than in mucosa ($P < 0.001$ two-tailed Mann–Whitney $U$ test). No bar for mucosa means that no FdUMP binding could be detected.**

absence and presence of NaF and CMP to inhibit nucleotide breakdown. We observed a 1.5–5 fold higher FdUMP binding; so the presence of NaF and CMP is essential. FdUMP binding was assayed in the presence of an optimum folate concentration. The amount of FdUMP binding in tumours varied between 0.1 and 1.0 (Fig. 3), being less than the variation in TS activity. Only in 2 patients (B and F) could FdUMP binding be detected in the absence of exogenous folate; binding was 5 fold lower. In mucosa FdUMP binding to TS was at least 3 times lower than that in tumour from the same patient, except patient L; FdUMP binding was always lower than 0.1 pmol/mg protein. In colonic mucosa binding of FdUMP was not detected in the absence of folates.

From 4 patients (C,E,F and H) from whom we obtained enough tumour material, intact cell preparations were prepared. Viability by trypan blue exclusion was 77% for the tumour cells and 51% for the colonocytes. In colonocytes incorporation of deoxyuridine into DNA could not be quantitated, in tumour cells incorporation of deoxyuridine into DNA varied between 0.03 and 0.11 pmol/h per $10^6$ cells. The ratio of deoxyuridine incorporation into DNA between patient C and F was similar to the ratio of TS activities. Unfortunately not enough material was available from the other patients to calculate such a ratio. After a preincubation with 1 or 10 μmol/l 5-FU, inhibition of deoxyuridine incorporation into DNA varying between 40 and 80 and 40 and 100%, respectively, was observed.

In most patients we were able to measure both TS catalytic activity and FdUMP binding capacity, which enabled us to calculate a ratio (Fig. 4). In mucosa these ratios varied from 96

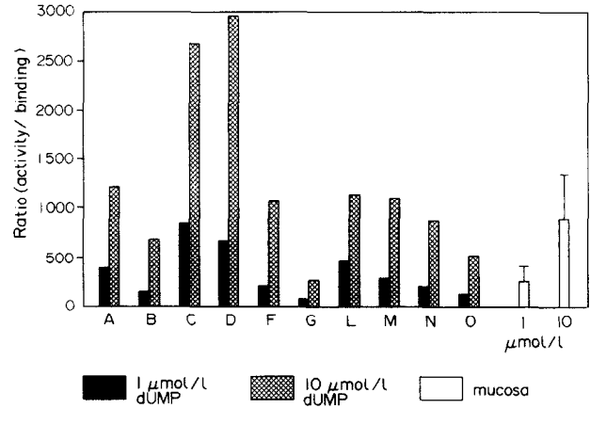


**Fig. 4. Ratio between TS catalytic activity (pmol/h per mg protein) and FdUMP binding capacity (pmol FdUMP bound per mg protein). Means and S.D. (open bars) are given for samples from normal mucosa assayed at 1 and 10 μmol/l dUMP. For tumours, ratios calculated from each patient are given. The ratios in tumours were not significantly different from those in mucosa.**

to 450 and from 368 to 1450 for TS catalytic activity at 1 and 10 μmol/l dUMP, respectively. However, in tumours these values varied from 75 to 837 and from 516 to 2961. Thus, in normal mucosa, all values were in a smaller range than those in tumours. The aberrant values in tumours were all higher than those in mucosa.

## DISCUSSION

In primary colorectal tumours TS showed a large variation in activity and inhibition by FdUMP. This biochemical heterogeneity may be related to variation in response. However, no data are available from the patients in this study to make such a correlation.

The activity of TS in tumours showed a much larger variation than in normal mucosa from the same patients. The small variation of TS in mucosa from these patients might be related to the normal interindividual differences, since similar differences have been observed in the activities of other enzymes involved in the activation of 5-FU [21, 26, 27]. However, in tumours from these patients, the activities of these enzymes also show a small variation, in contrast to that for TS. FdUMP binding to TS also had a large variation, but the pattern was different from that for TS activity. In the tumour with the lowest TS activity, we even found the highest FdUMP binding. TS activity expressed as deoxyuridine incorporation into DNA, and its inhibition by 5-FU, also showed considerable variation. However, this assay requires more material and is more laborious.

Variations in TS activity in colon tumours have also been observed by others [28–31]. Luccioni *et al.* [28] expressed this variation as the ratio in activity between tumour and normal tissue; the ratio varied from 0 to 24. The variation in TS activity could be partly related to aberrations in chromosomes, particularly in *n* under 18 [28, 32]. In contrast to Wolberg and Morin [29], we were able to measure TS activity in all samples of normal colon mucosa. TS activity in measurable samples of both mucosa and tumours reported by these investigators is in the same range as our values at 1 μmol/l dUMP. Results from Hashimoto *et al.* [31] are in the same range as our data at 10 μmol/l dUMP. Also, TS activity reported by Spears *et al.* [10] in pretreatment samples are similar to our data, as is inhibition by FdUMP. The number of FdUMP binding sites by Clark *et al.* [17] are similar to our findings. Danenberg and Bapat [30] described a variation in affinity of FdUMP for TS with $K_D$ values varying between 0.7 and 109 $\times$ $10^{-10}$, which could be mostly attributed to differences in the association of FdUMP with enzyme and not the dissociation. These data are in agreement with the observed differences in FdUMP binding sites.

We found a large difference in TS catalytic activity not only at a saturating dUMP concentration but also a variation in dUMP kinetics. The ratio between 10 and 1 μmol/l dUMP showed a larger variation than that observed between cell lines, especially in the presence of FdUMP [19]. Preclinical studies [19, 33] showed that inhibition by 10 nmol/l FdUMP at 1 and 10 μmol/l dUMP gives a good indication of the potential inhibition, without the necessity of detailed enzyme kinetic studies, which are not usually possible because of the limited quantities of clinical samples. Variations in kinetic properties of human TS from tumours for dUMP affinity have not been reported, in contrast to the situation for FdUMP binding [30]. The differences between tumours and mucosa concerning kinetic properties are greater when FdUMP inhibition at 1 and 10 μmol/l dUMP is compared and when the ratio between catalytic activity and FdUMP binding is taken into consideration. These data indicate that "mutant" TS enzymes may be present. The variation in potential inhibition by 10 nmol/l FdUMP, especially at 10 μmol/l dUMP in tumours, may have relevance for the outcome of treatment with 5-FU.

Measurement of TS at 10 and 1 μmol/l dUMP showed that intrinsic cytosolic dUMP levels were low, as has been observed in murine tumour and bone marrow [7]. Intrinsic dUMP does not interfere with the assay and 1 μmol/l dUMP is clearly suboptimal. Inhibition of TS by FdUMP at 10 μmol/l dUMP was still high, most likely because saturating levels of $CH_2$-THF were present in the assay. Under these conditions high dUMP would not affect binding of FdUMP to TS and inhibition of TS [34]. Physiological levels of $CH_2$-THF are low (0.1–2 μmol/l) as has been observed in xenografts from human colorectal cancer [13, 35], which explains the very low to undetectable FdUMP binding capacity in samples without added $CH_2$-THF. Thus, high $CH_2$-THF levels may abrogate dUMP effects.

These differences in TS activity and large variations in FdUMP binding and inhibition are in agreement with the differences observed *in vitro* between colorectal cell lines obtained from patients with different grades of resistance [18]. The kinetic differences may be the result of genetic and/or phenotypic variations, as expressed by differences in electrophoretic behaviour of the purified enzyme. The chromosomal aberrations [28, 32] indicate genetic variations. Several reports demonstrate that a high percentage (over 75%) of human tumours have a monosomic type loss of chromosome 18 [28, 32], which carries the gene for TS. Luccioni *et al.* [28] demonstrated that TS is low in this monosomic type of aberration and high in the trisomic type. It is not yet clear whether chromosomal abnormalities are also associated with deviations in kinetic properties of TS in patients. The large variation in TS activity may also be attributed to differences in expression of the TS gene. Evidence for gene amplification has been observed in 1 patient treated with 5-FU [17]; however no pretreatment sample of this patient could be analysed. So the difference in gene expression may even be related to a natural variation in TS.

The impact of the variation in TS activity and FdUMP inhibition in colorectal cancer on treatment efficacy is unknown. However, it is not unlikely that the difference in response to 5-FU between patients is related to variations in enzyme amount and properties. So, in several patients a higher concentration of FdUMP will be needed to achieve a required level of inhibition. Danenberg and Bapat [30] even postulated that these FdUMP levels should even be 100-fold higher. In tumour samples of patients, such a high variation in FdUMP has been observed [8–10], but the relation between FdUMP level and subsequent response to 5-FU treatment is not clear. Evidence is accumulating that there is a better relation between low pretreatment levels of TS, a high level of TS inhibition as a result of treatment and response to 5-FU treatment. Unfortunately, none of our patients described received subsequent 5-FU treatment.

Another interesting feature was the low inhibition of TS by FdUMP in normal mucosa, which is in agreement with the very low sensitivity of colon mucusa cells to 5-FU [36]; leucovorin enhanced this toxicity which is consistent with data on severe gastrointestinal toxicity of 5-FU plus leucovorin [7, 8]. Gastrointestinal toxicity in 5-FU treatment is schedule dependent, being lower with weekly bolus injections than with continuous 5 day infusions [37]. Since the physiological concentration of folates is low, TS inhibition would not be stabilised by endogenous folates and the enzyme would only be inhibited when

FdUMP is present at high concentrations for such a prolonged period.


1. Rode W, Kulikowski T, Kedzierska B, Jastreboff M, Shugar D. Inhibition of mammalian tumour thymidylate synthetase by 5-alkylated 2'-deoxyuridine 5'-monophosphate. *Biochem Pharmacol* 1984; **33**, 2699–2705.
2. Bapat AR, Zarov C, Danenberg PV. Human leukemic cells resistant to 5-fluoro-2'deoxyuridine contain a thymidylate synthase with lower affinity for nucleotides. *J Biol Chem* 1983, **258**, 4130–4136.
3. Danenberg PV. Thymidylate synthetase — a target enzyme in cancer chemotherapy. *Biochim Biophys Acta* 1977, **473**, 73–92.
4. Pinedo HM, Peters GJ. 5-fluorouracil: biochemistry and pharmacology. *J Clin Oncol* 1988, **6**, 1653–1664.
5. Creavan PJ, Petrelli NJ, Rustum YM. Leucovorin/5-fluorouracil: response and toxicity in relation to quality of life. In: Pinedo HM, Rustum YM, eds. *Leucovorin Modulation of Fluoropyrimidines: A New Frontier in Cancer Chemotherapy*. International Congress and Symposium Series No. 158, London, Royal Society of Medicine Services 1989, 57–64.
6. Nadal JC, Van Groeningen CJ, Pinedo HM, Peters GJ. Schedule-dependency of *in vivo* modulation of 5-fluorouracil by leucovorin and uridine in murine colon carcinoma. *Invest New Drugs* 1989, 7, 163–172.
7. Wright JE, Dreyfuss A, El-Magharbel I, *et al.* Selective expansion of 5,10-methylene-tetrahydrofolate pools and modulation of 5-fluorouracil antitumor activity by leucovorin *in vivo*. *Cancer Res* 1989, **49**, 2592–2596.
8. Peters GJ, Van der Wilt CL, Van Groeningen CJ, Nadal JC, Laurensse E, Pinedo HM. Development of leucovorin/5-fluorouracil combinations. In: Pinedo HM, Rustum YM, eds. *Leucovorin Modulation of Fluoropyrimidines: A New Frontier in Cancer Chemotherapy*. International Congress and Symposium Series No. 158. London, Royal Society of Medicine Services, 1989, 21–35.
9. Swain SM, Lippman ME, Egan EF, Drake JC, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989, 7, 890–899.
10. Spears CP, Gustavsson BG, Berne M, Frosing R, Bernstein L, Hayes AA. Mechanisms of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. *Cancer Res* 1988, **48**, 5894–5900.
11. Moore N, Ahmed F, Dunlap RB. Evidence for the existence of covalent nucleotide-thymidylate synthase complexes, identification of site of attachment, and enhancement by folates. *J Biol Chem* 1986, **261**, 12745–12749.
12. Davis ST, Berger SH. Genetic variation in thymidylate synthase structure is associated with 5-fluoropyrimidine resistance in human colonic tumor cells. [Abstr.] *Proc AACR* 1990, **31**, 422.
13. Houghton JA, Williams LG, Radparvar S, Houghton J. Characterization of the pools of 5,10-methylenetetrahydrofolates and tetrahydrofolates in xenografts of human colon adenocarcinoma. *Cancer Res* 1988, **48**, 3062–3069.
14. Yin MB, Zakrzewski SF, Hakala MT. Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. *Mol Pharmacol* 1983, **23**, 190–197.
15. Priest DG, Ledford SE, Doig MT. Increased thymidylate synthetase in 5-fluorodeoxyuridine resistant cultured hepatoma cells. *Biochem Pharmacol* 1980, **29**, 1549–1553.
16. Berger SH, Jenh CH, Johnson LF, Berger FG. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol Pharmacol* 1985, **28**, 461–457.
17. Clark JL, Berger SH, Mittelman A, Berger FG. Thymidylate synthase overproduction gene amplification in a colon tumor resistant to fluoropyrimidine chemotherapy. *Cancer Treat Rep* 1987, **71**, 261–265.
18. Berger SH, Barbour KW, Berger FG. A naturally occurring variation in thymidylate synthase structure is associated with a reduced response to 5-fluoro-2'-deoxyuridine in a human colon tumor cell line. *Mol Pharmacol* 1988, **34**, 480–484.
19. Peters GJ, Laurensse E, Van Groeningen CJ, Meijer S, Pinedo HM. *In vitro* and *in vivo* inhibition of thymidylate synthase of human colon cancer by 5-fluorouracil. *Adv Exp Med Biol* 1989, **25A**, 439–445.
20. 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.
21. Peters GJ, Laurensse E, Leyva A, Pinedo HM. Tissue homogenization using a microdismembrator for the measurement of enzyme activities. *Clin Chim Acta* 1986, **158**, 193–198.
22. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH. *In vivo* kinetics of thymidylate synthase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res* 1982, **42**, 450–456.
23. Pinkus LM, Separation and use of enterocytes. *Meth Enzymol* 1981, 77, 154–162.
24. Peters GJ, Veerkamp JH. Stimulation by phytohemagglutinin of peripheral blood lymphocytes from horse, pig, sheep and man. *Vet Immunol Immunopathol* 1982, **3**, 295–300.
25. Hashimoto Y, Shiotani T, Weber G. Simple separation of tritiated water and [$^3$H]deoxyuridine from 5-[$^3$H]deoxyuridine-5'-monophosphate in the thymidylate synthase assay. *Anal Biochem* 1987, **167**, 340–346.
26. Peters GJ, Laurensse E, Leyva A, Lankelma J, Pinedo HM. Differences in fluoropyrimidine metabolism between human colon tumors and colonic mucosa [Abstr.]. *Proc Amer Ass Cancer Res* 1985, **26**, 12.
27. Peters GJ, Van Groeningen, Laurensse E, Pinedo HM. A comparison of 5-fluorouracil metabolism in human colorectal cancer and colon mucosa. *Cancer* 1991, (in press).
28. Luccioni C, Muleris M, Sabatier L, Dutrillaux B. Chromosomal and enzymatic patterns provide evidence for two types of human colon cancers with abnormal nucleotide metabolism. *Mutation Res* 1988, **200**, 55–62.
29. 29. Wolberg WH, Morin J. Thymidylate synthetase activity and fluorouracil sensitivity of human colonic cancer and normal mucosal tissue preparations. *Cancer* 1981, **47**, 1313–1317.
30. Danenberg PV, Bapat AR. Thymidylate synthases isolated from human colon tumours have varied affinity towards 5-fluoro-2'-deoxyuridylate. *Reg Cancer Treat* 1989, **2**, 1–4.
31. Hashimoto Y. Shiotani T, Eble JN, Glover JL, Weber G. Increased thymidylate synthase (EC 2.1.1.45) activity in normal and neoplastic proliferation. *Cancer Biochem Biophys* 1988, **10**, 1–10.
32. Vogelstein B, Fearon ER, Kern SE, *et al.* Allelotype of colorectal carcinomas. *Science* 1989, **244**, 207–211.
33. Peters GJ, Laurensse E, Leyva A, Pinedo HM. Purine nucleosides as cell-specific modulators of 5-fluorouracil metabolism and cytotoxicity. *Eur J Cancer Clin Oncol* 1987, **23**, 1869–1881.
34. Spears CP, Hayes AA, Shahinian AH, *et al.* Deoxyuridylate effects on thymidylate synthase-5-fluorodeoxyuridylate-folate ternary complex formation. *Biochem Pharmacol* 1989, **38**, 2985–2993.
35. Houghton JA, Schmidt C, Houghton PJ. The effect of derivatives of folic acid on the fluorodeoxyuridylate-thymidylate synthase covalent complex in human colon xenografts. *Eur J Cancer Clin Oncol* 1982, **18**, 347–354.
36. Au J L-S, Bramer SI. Toxicity of fluoropyrimidines and leucovorin to rat small intestinal epithelial cells [Abstr.]. *Proc AACR* 1990, **31**, 426.
37. Seifert P, Baker L, Reed ML, *et al.* Comparison of continuously infused 5-fluorouracil with bolus injection in treatment of patients with colorectal adenocarcinoma. *Cancer* 1975, **36**, 123–128.


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