novel target for inhibiting gastric cancer cell invasion. *J Natl Cancer Inst* 1993, 85, 39–46.
40. Rodriguez-Pena A, Rozengurt E. Disappearance of Ca^{2+} sensitive phospholipid dependent protein kinase activity in phorbol ester-treated 3T3 cells. *Biochem Biophys Res Comm* 1980, **120**, 1053–1059.
41. Kraft AW, Anderson WB. Phorbol ester increase the amount of CA^{++}, phospholipid dependent protein kinase associated with plasma membrane. *Nature* 1983, **301**, 621–623.
42. Giraldi T, Perissin L, Zorzet S, Rapozzi V. Antimetastatic action of triazene derivatives. In Giraldi T, Connors TA, Cartei G, eds. *Triazenes*. New York, Plenum Publishing, 1990, 45–62.
43. Heyes J. Antimetastatic effect of 4 carbethyoxy-5(3,3-diethyl- 1 triazeno)-2-methylimidazole. *J Natl Cancer Inst* 1974, **53**, 279–285.
44. Giraldi T, Hougton PJ, Taylor DM, Nisi C. Antimetastatic action of some triazene derivatives against the Lewis lung carcinoma in mice. *Cancer Treatment Rep* 1978, **62**, 721–725.
45. Sava G, Giraldi T, Zupi G, Sacchi A. Effects of antimetastatic dimethyltriazenes in mice bearing Lewis lung carcinoma lines with different metastatic potential. *Invasion Metastasis* 1984, **4**, 171–178.
46. Zupi G, Corsi A, Sacchi A, Lassiani L, Giraldi T. Effects of dimethyltriazenes on *in vitro* Lewis lung carcinoma tumour lines with different metastatic capacity. *Invasion Metastasis* 1984, **4**, 179–180.
47. Bonmassar E, Bonmassar A, Vadlamudi S, Goldin A. Immunological alteration of leukemic cells *in vivo* after treatment with an antitumour drug. *Proc Natl Acad Sci USA* 1970, **66**, 1089–1095.
48. Allegrucci M, Fuschiotti P, Puccetti P, Romani L, Fioretti MC. Changes in the tumorigenic and metastatic properties of murine melanoma cells treated with a triazene derivative. *Clin Exp Metastasis* 1989, **3**, 329–341.
49. Martin-Padura I, Mortarini R, Lauri D, *et al.* Heterogeneity in human melanoma cell adhesion to cytokine activated endothelial cells correlates with VLA-4 expression. *Cancer Res* 1991, **51**, 2239–2241.
50. Dahl SC, Grabel LB. Integrin phosphorylation is modulated during the differentiation of F-9 teratocarcinoma cells. *J Cell Biol* 1989, **108**, 183–190.
51. Chatila TA, Geha RS, Arnaut MA. Constitutive and stimulus induced phosphorylation of CD11/CD18 leukocytes adhesion molecules. *J Cell Biol* 1989, **109**, 3345–3444.
52. Dehar S, Saulnier R. Alteration in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. *J Cell Biol* 1990, **110**, 481–489.
53. Liotta LA, Rao CN, Wever UM. Biochemical interactions of tumor cells with the basement membrane. *Annu Rev Biochem* 1986, **55**, 1037–1057.
54. Dehar S. Integrins and tumor invasion. *Bioassays* 1990, **12**, 583–590.

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The Effect of Different Routes of Administration of 5-Fluorouracil on Thymidylate Synthase Inhibition in the Rat

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A rat colon tumour model of liver metastases was used to administer 5-fluorouracil (5FU) by intraperitoneal (i.p.) bolus injection (50 mg/kg), isolated liver perfusion (ILP, 150 mg/kg) and hepatic artery infusion (HAI, 50 mg/kg). The biochemical effect of 5FU, delivered by different routes, on its target enzyme thymidylate synthase (TS) was studied in both tumour and normal tissues of the rat. In tumour tissue, only small differences were observed in the extent of TS inhibition. A pronounced inhibition of TS was observed 3 h after 5FU administration by all routes, but was followed by a recovery of TS activity within 24 and 48 h. Effects of 5FU on normal tissues were diverse. In liver, TS activity increased 6-fold after ILP and HAI administration of 5FU, and a 2-fold increase of FdUMP binding to TS was seen for all routes of administration. In intestinal mucosa, both induction of TS activity (by ILP) and inhibition of TS activity (by HAI) were observed, while i.p. injection did not cause major changes. TS activity and FdUMP binding to TS in bone marrow was strongly inhibited after administration of 5FU by all routes, but administration by ILP seemed slightly advantageous, since a smaller extent of TS inhibition was observed compared to the other routes of administration. 5FU given by ILP had a small antitumour effect in this colon tumour model, while HAI administration had no antitumour activity. Since this difference in antitumour activity could not be related to differences in TS inhibition in the tumour, the RNA-directed mechanism of action of 5FU could be involved. Focusing on the effects of TS, we may conclude that the ILP administration of 5FU offered the important advantage of a lack of severe TS inhibition in normal tissues, which corresponds with the low systemic toxicity observed.

Key words: thymidylate synthase inhibition, 5-fluorouracil, route of administration, animal model, rat, liver carcinogenesis

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INTRODUCTION

5-FLUOROURACIL (5FU) is used for the treatment of several types of human cancers, either as a single agent or in combination with other drugs. Its biochemical effects that finally cause cell kill are related to three main metabolites, FdUMP, FUTP and FdUTP. FUTP and FdUTP can be misincorporated into RNA and DNA, respectively. FdUMP is a potent inhibitor of thymidylate synthase (TS), an essential enzyme in the process of DNA synthesis [1].

The antitumour effect of fluoropyrimidines, such as 5FU, is dependent on dose and duration of exposure. Dose and time dependence has been shown in experimental model systems [2–4] and in patients [5, 6]. *In vitro* resistance against 5FU could be overcome by using high concentrations of 5FU, up to 20–40 fold higher than those clinically achievable during systemic therapy [7]. The toxic side effects of 5FU in patients depend on dose, schedule and route of administration of the drug. For bolus injections, bone marrow toxicity is the dose limiting toxicity, whereas continuous infusion is limited by gastrointestinal toxicity [8].

Regional treatment allows the use of higher doses of drug than that used in systemic treatment, without increase in toxicity [9]. It is often applied to expose liver metastases to higher doses of fluoropyrimidines. 5FU is one of the drugs suitable for regional administration due to its favourable extraction ratio [10, 11]. The high catabolism for 5FU in the liver allows administration of a high dose without increasing systemic plasma concentrations [12]. The most common route of regional administration is hepatic artery infusion (HAI). Often, 2'-deoxy-5-fluorouridine, a more potent fluoropyrimidine, is used for HAI [13,14]. This compound has an even higher extraction ratio than 5FU [10]. Toxic side effects of this method of administration of fluoropyrimidines include gastrointestinal symptoms, hepatobilary toxicity and less frequently myelosuppression [15, 16]. After 5FU administration by HAI, no serious hepatic toxicity has been reported [17].

In a rat model, in which liver metastases from syngeneic colorectal cancer could be induced, an isolated liver perfusion (ILP) technique was developed to allow exposure of the tumour to still higher concentrations, while systemic exposure associated with systemic toxicity remained minimal. ILP allowed administration of a 3-fold higher dose of 5FU than tolerated with HAI [18, 19], but it required a complicated surgical procedure [20]. Currently, patients with irresectable colorectal cancer metastases confined to the liver have been treated with ILP in a phase II study [21]. Systemic toxicity of ILP was minimal, but the risk of complications due to surgical procedures was higher compared with systemic or intraperitoneal (i.p.) administration. The high dose of drug caused some hepatotoxic side effects, monitored by transient elevation of alkaline phosphatase (AP), glutamic oxaloacid transaminase (SGOT) and glutamic pyruvatic acid transaminase (SGPT) levels [22].

The aim of this study was to determine whether regional 5FU administration, either by ILP or HAI, had a different biochemical effect in terms of inhibition of TS than i.p. administration. The value of TS and TS inhibition as important parameters in the evaluation of 5FU treatment has been indicated in different studies [23–28]. The 5FU-mediated inhibition of TS was measured in both tumour and normal tissues in order to give an insight into the benefits of selective high dose administration of 5FU into the liver. Additionally we studied the antitumour activity of regional 5FU administration on liver metastases in the rat.

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METHODS

Materials

5FU was obtained from Hoffman-La Roche (Mijdrecht, The Netherlands). [6-^{3}H]-FdUMP (specific activity 20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, California, U.S.A.) and [5-^{3}H]-dUMP (specific activity 10.9 Ci/mmol) from Amersham International (Buckinghamshire, U.K.). All other chemicals were of analytical grade, and were commercially available.

Rats

Male WAG/Ola rats (Harlan/Olac; C.P.B., Zeist, The Netherlands), 3 months of age, weighing approximately 300 g were used for this study. Four/five animals were housed per cage, and they received food and water *ad libitum*. The selected tumour line, CC531, was derived from a dimethylhydrazine-induced, moderately differentiated, colon cancer [29]. It was cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin. Liver metastases were induced by subcapsular injection of 5×10^5 cells in 0.05 ml 0.9% NaCl into the right and left main lobe of the liver, and single tumours formed in each lobe. Injection of cells via the portal vein gave rise to many micrometastases. Ten days after inoculation, tumours with a cross sectional area of about 40 mm^2 were visible.

Regional administration

Two different routes of administration were used, HAI and ILP which were applied as described previously [19, 30]. 5FU was given at the maximal tolerated dose (MTD) for each route of administration in these rats, 50 mg/kg for HAI and 150 mg/kg for ILP. Treatment was given once. The protocol was approved by the institutional ethical committee.

For HAI, the gastroduodenal artery, a branch of the common hepatic artery, was cannulated. During the infusion of 50 mg/kg 5FU, the common hepatic artery was clamped to prevent retrograde flow into the aorta. Infusion time was approximately 2 min [30].

ILP in the rat was performed as described [19, 30]. The inflow of the isolated circuit was by cannulas in the pyloric branch of the portal vein and in the gastroduodenal branch of the common hepatic artery, while a cannula inserted in the caval vein served as outflow. The circuit was isolated by clamping the caval vein above and below the liver, the aorta above the coeliac axis and the common hepatic artery and the portal vein just below the tips of the cannulas. A dose of 150 mg/kg 5FU was injected into the isolated circuit and the perfusate was recirculated for 25 min.

Thymidylate synthase assays on tissues

The effect of 5FU therapy was analysed biochemically at the level of TS: TS inhibition following regional 5FU therapy was compared with the effect of systemic administration. The systemic treatment consisted of a bolus injection (i.p.) of 50 mg/kg 5FU, which is the MTD for weekly treatment. Forty rats

were used for this study. At least 3 rats were used per time point for each route of administration. Tumour, liver, intestinal mucosa and bone marrow cells of the rats were removed 3, 24 and 48 h after treatment and immediately frozen in liquid nitrogen. Samples from untreated rats served as controls. Tissues were homogenised with a microdismembrator (Braun, Melsungen, Germany) as described previously [31], and suspended in a buffer of 0.2 M TRIS/HCl (pH 7.4), 20 mM mercaptoethanol, 100 mM NaF and 15 mM CMP. In the samples from treated animals, all free FdUMP was absorbed with 10% neutral charcoal before TS assays were performed.

TS inhibition was evaluated with two assays, a ligand binding assay, which determined the free binding sites for FdUMP, and a ^{3}H-release assay to determine the catalytic activity of TS (conversion of dUMP into dTMP) as described [32, 33]. The ligand binding assay was performed with [6-^{3}H]-FdUMP as a substrate. Briefly, 50 μl enzyme suspension was incubated with 50 μl 6.5 mM 5,10-methylenetetrahydrofolate, 135 μl TRIS/HCl buffer and 10 μl 0.57 μM [6-^{3}H]-FdUMP for 1 h at 37 °C. The reaction was stopped by the addition of 500 μl 10% neutral charcoal and 250 μl of the supernatant was used for radioactivity counting. Detection limit for this assay was 45 pmol/mg protein.

For the ^{3}H-release assay, [5-^{3}H]-dUMP was used as a substrate. Two concentrations of dUMP was used in this assay, 1 μM which is around the Km, and 10 μM a saturating substrate concentration. Two substrate concentrations were used because the ratio between the catalytic activity at 1 and 10 μM dUMP shows whether the substrate specificity and affinity characteristics of the enzyme are preserved during 5FU treatment. Briefly, for this assay, 25 μl enzyme suspension (at different dilutions) were incubated with 5 μl 6.5 mM 5,10-methylenetetrahydrofolate, 10 μl TRIS/HCl buffer and 10 μl [5-^{3}H]-dUMP (1 or 10 μM final concentration) for 30 min at 37 °C. In control samples, we measured the potential inhibition of TS by adding 10 μl 0.05 μM FdUMP, instead of the TRIS/HCl buffer. The assay was stopped by addition of 50 μl ice-cold 35% trichloroacetic acid and 250 μl 10% neutral charcoal, and 150 μl of the supernatant was used for radioactivity counting by liquid scintillation. The detection limit of this assay was 2.5 pmol/h/mg protein. Protein content of the samples was measured using Coomassie-blue (Bio-Rad, Veenendaal, The Netherlands).

Antitumour effect

Tumour bearing rats with two tumours were randomly assigned to 5 groups: (1) untreated control (n = 8), (2) HAI without drug (n = 4), (3) ILP without drug (n = 4), (4) HAI with 50 mg/kg 5FU (n = 8), (5) ILP with 150 mg/kg 5FU (n = 6). When the 5FU dose regionally delivered was elevated (higher than the MTD used for these experiments), rats died of systemic toxicities (low white blood cell counts, diarrhoea, pneumonia). The antitumour effect of 5FU was evaluated on days 0 (day of treatment, 10 days after tumour inoculation), 14, 28 and 42. Rats were weighed and, in order to measure liver tumours, laparotomy was performed. Cross sectional areas of the tumours were estimated by calliper measurements, and calculated as $\pi \times 0.25 \times$ maximal diameter $\times$ perpendicular diameter [34]. Rats were sacrificed at day 42 because the tumours in untreated groups became too large (>500 mm^2). Toxicity parameters studied included survival, weight, serum levels of SGOT, SGPT, AP and bilirubin. At day 3 after drug administration, rats were sampled by retro-orbital puncture under ether anaesthesia to collect serum for the blood chemistry. This was repeated at day 7 and weekly thercafter.

Statistics

The antitumour effects were evaluated using the Mann–Whitney U test. The FdUMP binding data and results on catalytic activity after 5FU exposure were evaluated with Student's t-test for unpaired data.

RESULTS

Biochemical analysis of TS levels in control tissues, measured with the FdUMP binding assay and the ^{3}H-release assay (Table 1), showed clearly that TS levels in tumour tissue were much higher than in liver and intestinal mucosa. Comparison of control tumour and control bone marrow revealed that FdUMP binding and TS catalytic activity in bone marrow was higher than in tumour. The potential inhibition by 10 nM FdUMP, added to control samples, was approximately 60–70% for all tissues, when measured at 10 μM dUMP and even higher when measured at 1 μM dUMP. At 1 μM dUMP, the TS inhibition was less pronounced in intestinal mucosa compared with other tissues.

The various routes of 5FU administration caused different effects on TS levels in tumour and normal tissues of the rat. Both an elevation and an inhibition of TS were observed. FdUMP binding to TS in tumour tissue was inhibited at 48 h, irrespective of the route of administration. All values, except HAI 48 h, were significantly lower than control values ($P < 0.001$). The most pronounced inhibition was observed 3 h after i.p. administration. No notable differences in the retention of TS inhibition were observed for the different routes of administration, as measured by FdUMP binding or TS catalytic activity. The maximal extent and retention of the inhibition of TS catalytic activity (Figure 1a) in tumour tissue was less than if measured by FdUMP binding. The maximal extent was 57% compared with 85% for FdUMP binding. In contrast to FdUMP binding, TS catalytic activity, measured at 1 μM dUMP substrate concentration, had completely recovered 48 h after 5FU by ILP or i.p., and had nearly recovered after HAI. Inhibition of TS catalytic activity, measured at 10 μM dUMP (not shown), was more pronounced than at 1 μM and recovered more slowly.

In liver tissue, no significant inhibition of TS was found for either route of administration with the FdUMP binding assay (Table 2). The more sensitive ^{3}H-release assay showed that the TS activity 3 h after treatment was lower than control values (Figure 1b). The increase of TS activity and FdUMP binding, observed at 24 and 48 h after treatment was striking (Table 2, Figure 1b). This was most pronounced for HAI and ILP administration of 5FU, with a more than 6-fold increase of TS activity in the liver.

We could not detect significant changes in FdUMP binding in intestinal mucosa between control and treated samples nor between ILP, i.p. and HAI administration, due to variation (Table 2). Evaluation of the catalytic activity (Figure 1c) showed a clear 3-fold increase of activity for administration by ILP at 48 h, while HAI caused a 5-fold decrease. Values of samples obtained after i.p. administration were not significantly different from control.

TS activity and FdUMP binding in bone marrow cells were markedly decreased by 5FU treatment. Administration of 5FU by ILP resulted in less inhibition of TS in bone marrow than HAI or i.p. administration, but the differences were only marginal (Table 2, Figure 1d).

The antitumour effect of regional administration of 5FU was very small (Table 3). HAI administration of 5FU had no antitumour activity and ILP delivery had only slight antitumour activity (Table 3). Tumour growth for animals that underwent

Table 1. FdUMP binding and TS catalytic activity in untreated rat tissues

	Tumour	Liver	Intestinal mucosa	Bone marrow cells*
FdUMP binding	34.4 ± 4.6†	9.2 ± 0.7†	8.6 ± 6.1†	110 ± 13‡
TS catalytic activity				
at 1 μM dUMP	215 ± 115§	36 ± 9§	55 ± 31§	154 ± 57¶
+ 10 nM FdUMP	51 ± 29§	1.5 ± 1§	23 ± 4§	21 ± 5¶
at 10 μM dUMP	961 ± 271§	119 ± 33§	206 ± 65§	1078 ± 169¶
+ 10 nM FdUMP	321 ± 92§	45 ± 6§	88 ± 62§	353 ± 32¶
Protein content	60‖	125‖	50‖	25**

* 10^6 cells = 1 mg wet weight; Values are means ± SD of 3–5 experiments, † pmol/g wet weight, ‡ fmol/10^6 cells, § pmol/h/mg protein, ¶ pmol/h/10^6 cells, ‖ mg protein/g wet weight, ** μg protein/10^6 cells.

Table 2. Inhibition of FdUMP binding to TS in rat colon tumour and normal tissues after administration of 5FU by different routes

t (h)	HAI (50 mg/kg)	ILP (150 mg/kg)	i.p. (50 mg/kg)	t (h)	HAI (50 mg/kg)	ILP (150 mg/kg)	i.p. (50 mg/kg)
Tumour	(pmol/g wet weight) (n)			Liver	(pmol/g wet weight) (n)		
0	34.4 ± 4.6 (5)	34.4 ± 4.6 (5)	34.4 ± 4.6 (5)	0	9.2 ± 0.7 (3)	9.2 ± 0.7 (3)	9.2 ± 0.7 (3)
3	7.6 ± 2.9 (4)	7.7 ± 0.7 (4)*	5.3 ± 0.8 (4)*	3	5.9 ± 2.7 (3)	7.7 ± 3.5 (3)	11.0 ± 3.3 (3)
24	19.7 ± 4.3 (3)	14.2 ± 8.6 (3)	14.4 ± 3.0 (5)	24	7.8 ± 1.4 (5)†	15.8 ± 7.3 (5)	17.2 ± 3.2 (3)†
48	22.0 ± 11.1 (3)	17.4 ± 6.9 (4)	11.4 ± 6.2 (3)	48	10.7 ± 2.6 (4)	17.7 ± 8.5 (4)	13.7 ± 5.8 (3)
Intestinal mucosa	(pmol/g wet weight) (n)			Bone marrow cells	(pmol/10^6 cells) (n)		
0	8.6 ± 6.1 (5)	8.6 ± 6.1 (5)	8.6 ± 6.1 (5)	0	110 ± 13 (3)	110 ± 13 (3)	110 ± 13 (3)
3	15.5 ± 9.9 (3)	8.1 ± 4.2 (3)	6.2 ± 2.7 (3)	3	31 ± 17 (3)	57 ± 23 (3)‡	22 ± 3 (3)‡
24	4.4 ± 3.0 (4)	4.9 ± 3.4 (4)	6.8 ± 3.8 (3)	24	17 ± 7 (4)	21 ± 13 (4)	14 ± 4 (3)
48	2.5 ± 1.8 (4)	7.6 ± 6.8 (3)	4.8 ± 2.5 (3)	48	4 ± 1 (3)§	13 ± 8 (4)§	3 ± 1 (3)§

Values are means ± SD of 3–5 experiments. Statistics: * 3 h after 5FU administration, there was a significant difference in FdUMP binding to TS in tumour tissue between ILP and i.p. ($p < 0.01$). † At 24 h after HAI administration of 5FU, FdUMP binding in liver was significantly different from FdUMP binding after i.p. administration ($p < 0.01$). ‡ 3 h after ILP, FdUMP binding to TS in bone marrow was significantly higher than after i.p. administration. § 48 h after ILP, FdUMP binding was significantly higher in bone marrow than after i.p. or HAI administration ($P < 0.03$).

Table 3. Antitumour activity and toxicity of regional 5FU administration

Days‡	Relative cross sectional area of tumours* Control	HAI	ILP	Weight changes (g)† Control	HAI	ILP
0				+21	+13	+13
14	3.78 ± 1.12	4.21 ± 1.29	2.52 ± 0.90§	+21	+13	+13
28	9.40 ± 2.91	8.39 ± 2.13	6.42 ± 4.18	+24	+18	+15
42	15.03 ± 4.18	13.32 ± 3.91	11.43 ± 7.01	+25	+22	+22

* Values are the ratio to control values, and are means ± SD of 6 (ILP) or 8 (control, HAI) animals. Doses of 5FU by HAI and ILP were 50 and 150 mg/kg, respectively. Results were expressed relative to the initial surface of each tumour, thereafter the means were calculated (absolute initial values: control 27 mm^2, coefficient of variation (CV) 50%; HAI 37 mm^2, CV 40%; ILP 46 mm^2, CV 50%. † Weight at tumour inoculation was approximately 300 g. ‡ Days after treatment. § Significant reduced tumour growth compared to control ($P < 0.05$).

HAI or ILP without drug was comparable with controls (data not shown). Weight changes after treatment were positive, indicating that no long-term 5FU therapy-associated weight loss occurred (Table 3). Regional administration of 5FU, either by HAI or by ILP, did not result in increases of SGOT, SGPT, AP or bilirubin (data not shown), so 5FU did not cause hepatic toxicity.

DISCUSSION

The biochemical efficacy of 5FU, evaluated at the level of TS inhibition in tumour tissue, showed no notable differences between ILP (150 mg/kg), HAI (50 mg/kg) and i.p. (50 mg/kg) administration. A marked inhibition of TS was observed 3 h after treatment, but within 24 and 48 h both TS catalytic activity and FdUMP binding had recovered.

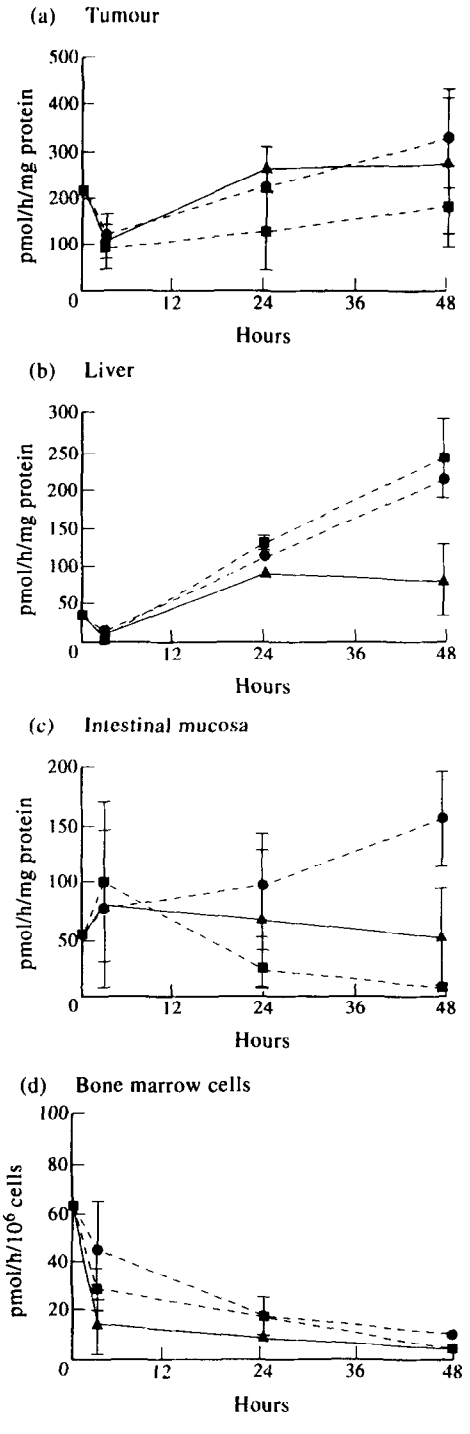

Figure 1. Comparison of residual TS catalytic activity (at 1 μM dUMP) after ILP (150 mg/kg 5FU) (- ● -), HAI (50 mg/kg 5FU) (- ■ -) and i.p. (50 mg/kg 5FU) (- ▲ -) treatment in the rat. Values are means ± SD, a small SD is within the symbol, $n = 3$–6. (a) A significant difference was observed between TS activity in tumour tissue at 24 h after HAI and i.p. administration of FU ($P < 0.01$). (b) Results of TS activity after ILP and HAI delivery differed significantly from those after i.p. injection of FU at 24 and 48 h ($P < 0.05$). (c) ILP administration resulted in significantly higher TS activity in intestinal mucosa at 48 h than the activity measured after i.p. or HAI administration ($P < 0.05$). TS activity in intestinal mucosa was also significantly higher 24 h after ILP administration compared to HAI ($P < 0.02$). (d) At 3 h, the residual TS activity in bone marrow cells was significantly higher after ILP than after HAI or i.p. administration ($P < 0.01$) and ($P < 0.02$), respectively.

Unlike tumour tissue, TS in the liver of the rat was hardly affected by 5FU. Comparison of different routes of 5FU delivery showed that both regional delivery and i.p. administration resulted in a minor inhibition of TS in the liver. On the contrary, an increase of TS catalytic activity and FdUMP binding to TS were observed after 24 h. FdUMP binding values were given per g wet weight; this showed that the increase was not due to an overall increase in proteins. In addition, the protein content per g wet weight remained constant.

Increase of TS after 5FU administration has also been observed in tumour tissue of other colon tumour models [33, 35]. In a murine colon tumour, the increase of TS activity after repeated 5FU treatment seemed to account for resistance of the tumour against 5FU [33]. Studies of Chu and associates [36] showed that the elevation of TS in a colon carcinoma cell line after exposure to 5FU was related to an increased translation of TS mRNA. The translation of TS mRNA is autoregulated by its own protein, which binds to TS mRNA and blocks translation. However, when the protein interacts with FdUMP (or dUMP, or reduced folate), it can no longer bind to the mRNA and uncontrolled enzyme synthesis occurs [37]. The liver and other normal tissues have a strictly regulated metabolism, and therefore enzyme levels are low compared with tumour tissue [38]. FdUMP binding to TS and TS catalytic activity were higher, by approximately 3- and 6-fold, respectively, in tumours of untreated animals compared with in liver and gut mucosal tissue. Tumours already have an irregular metabolism, and may be less sensitive to regulatory factors, such as the autoregulation of TS mRNA translation. Thus, even a small deregulation of TS mRNA translation in liver will have a relatively greater effect than in the tumour, leading to the observed increase in TS activity. Deregulation of TS synthesis in the presence of FdUMP (derived from 5FU), leading to an elevation of TS activity and possibly also of TS protein, could be a more general mechanism to reduce the stress on cells caused by the inhibition of TS. This hypothesis of an effective increase of TS, which would diminish the sensitivity of liver cells for 5FU-mediated TS inhibition, was supported by the observation that, even at high concentrations of 5FU in the liver, such as occur at HAI (0.30 μmol/g) and ILP (4.65 μmol/g) administration, liver toxicity in the rats was absent. 5FU concentrations in liver tissue, measured 15 min after administration, were higher than in tumour tissue (0.30 versus 0.20 μmol/g for HAI and 4.65 versus 1.06 μmol/g for ILP [unpublished data]). Since initial 5FU levels determine the FdUMP levels [11], more FdUMP will be formed in the liver, which binds to TS protein, thereby interrupting the autoregulation of TS mRNA.

ILP caused a similar increase of TS activity in the intestinal mucosa as was seen in the liver, while HAI caused inhibition of TS activity in the intestinal mucosa. Administration of 5FU by HAI might be accompanied by leakage of a high concentration of 5FU to the blood supplying vessels of the gastrointestinal track if the extraction of 5FU by the liver is incomplete. The latter is not unlikely due to the rapid administration of this dose of 5FU. In patients HAI of 5FU is usually applied over a longer period, e.g. 5 days [28], resulting in a better extraction. For ILP, the extraction was possibly better because the circulation of the liver was isolated from the systemic circulation; this precluded high concentrations of 5FU introduced into the liver leaking into the systemic circulation. It has been shown that 5FU plasma levels in the systemic circulation were significantly lower for ILP than for HAI [18]. The latter also explains the greater extent of TS inhibition in bone marrow cells after HAI

and i.p. compared with ILP. For ILP administration, a 3-fold higher dose of 5FU was used than for i.p. and HAI. This implied that, with ILP administration, tumour cells could be exposed to higher concentrations of 5FU, without an increase of bone marrow toxicity.

It is hard to predict whether high concentrations of 5FU and subsequently of FdUMP will cause prolonged inhibition of TS or an increase of TS, due to deregulation of TS mRNA translation. It is not yet clear at which 5FU/FdUMP concentration deregulation or increase of TS prevails, or whether the TS level of the tissue plays a role. Probably we observed a dual effect in most tissue samples, and in some of them one of the two effects prevailed, in liver the deregulation and in bone marrow the inhibition. Recently developed methods to measure TS protein [39, 40] and TS mRNA [36, 41] could give more information on deregulation of TS protein synthesis.

The antitumour activity study showed that the effect of regional and i.p. 5FU administration against this tumour was rather limited. Initially, a significant difference in tumour cross sectional area between control and ILP was observed. This difference was lost during the experiment, when the growth rate of tumours of ILP treated animals was comparable with the control. This showed one of the disadvantages of ILP, in that the treatment is given only once. HAI delivery allows repeated administration of 5FU, therefore ILP is often combined with HAI when used in the clinic [42]. Several factors could play a role in the 5FU resistance. Firstly, it has been shown that the sensitivity to 5FU of CC531 transplantable rat colon tumour is highly dependent on the tumour site [29, 43, 44]. Secondly, there was no relation between the initial antitumour effect obtained with ILP and the extent or retention of TS inhibition in the tumour, since the latter was similar for all administration routes. The other mechanism of action of 5FU, incorporation of FUTP into RNA, might be more important for the antitumour effect of 5FU administration by ILP. Thirdly, it is known that thymidine levels in the plasma of rodents are rather high compared with humans [45]. This may reduce the antitumour effect of 5FU related to TS inhibition.

This study showed, despite the low antitumour activity of 5FU against this tumour, that regional treatment by ILP has several benefits. The 3-fold higher dose that could be administered, compared with HAI and i.p., achieved at least some antitumour activity, although this was not biochemically associated with a greater extent of TS inhibition in the tumour. The high dose did not cause an increase of TS inhibition in normal tissues in and outside the liver, which seems to correspond to the resistance of liver tissue and the low systemic toxicity of this route of administration. ILP may therefore represent a promising therapy for metastasis confined to the liver.

1. Weckbecker G. Biochemical pharmacology and analysis of fluoropyrimidines alone and in combination with modulators. *Pharmac Ther* 1991, **50**, 367–424.
2. Frei III E, Canellos GP. Dose: A critical factor in cancer chemotherapy. *Am J Med* 1980, **69**, 585–593.
3. Drewinko B, Yang LY. Cellular basis for the inefficacy of 5-FU in human colon carcinoma. *Cancer Treat Rep* 1985, **69**, 1391–1398.
4. Van Ark-Otte J, Peters GJ, Pizao PE, Keepers YPAM, Giaccone G. *In vitro* schedule-dependency of EO9 and Miltefosine in comparison to standard drugs in colon cancer cells. *Int J Oncol* 1994, **4**, 709–715.
5. Hryniuk WM, Figueredo A, Goodyear M. Applications of dose intensity to problems in chemotherapy of breast and colorectal cancer. *Semin Oncol* 1987, **14**, 3–11.
6. Lokich JJ, Ahlgren JC, Gullo JJ, Philips JA, Fryer JG. A prospective randomized comparison of continuous infusion fluorouracil with a conventional bolus schedule in metastatic colorectal carcinoma: A Mid-Atlantic Oncology Program study. *J Clin Oncol* 1989, **7**, 425–432.
7. Drewinko B, Yang LY, Ho DWH, Benevenuto J, Loo TL, Freireich EJ. Treatment of cultured human colon carcinoma cells with fluoropyrimidines. *Cancer* 1980, **45**, 1144–1158.
8. Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokin* 1989, **16**, 215–237.
9. Wagner JG, Gyves JW, Stetson PL, Walker-Andrews SC, Wollner IS, Cochran MK, Ensminger WD. Steady-state nonlinear-pharmacokinetics of 5-fluorouracil during hepatic arterial and intravenous infusions in cancer patients. *Cancer Res* 1986, **46**, 1499–1506.
10. Frei III E, Garnick MB, Ensminger WD, *et al.* Biochemical pharmacology in medical oncology. *Cancer Treat Rep* 1981, **65**, 21–26.
11. Peters GJ, Lankelma J, Kok RM, *et al.* Prolonged retention of high concentrations of 5-fluorouracil in human and murine tumors as compared with plasma. *Cancer Chemother Pharmacol* 1993, **31**, 269–276.
12. Ensminger WD, Rosowsky A, Raso V, *et al.* A clinical-pharmacological evaluation of hepatic arterial infusions of 5-fluoro-2′-deoxyuridine and 5-fluorouracil. *Cancer Res* 1978, **38**, 3784–3792.
13. Kemeny N, Daly J, Reichamn B, Geller N, Botet J, Oderman P. Intrahepatic artery or systemic infusion of fluorodeoxyuridine in patients with metastases from colorectal carcinoma. *Ann Int Med* 1987, **107**, 459–465.
14. van de Velde CJH, De Brauw LM, Sugarbaker PH, Tranberg KG. Hepatic artery infusion chemotherapy: rationale, results, credits and debits. *Reg Cancer Treat* 1988, **1**, 93–101.
15. Hohn DC, Rayner AA, Economou JS, Ignoffo RJ, Lewis BJ, Stagg RJ. Toxicity and complications of implanted pump hepatic arterial and intravenous floxuridine infusion. *Cancer* 1986, **57**, 465–470.
16. Hohn DC, Melnick J, Stagg R, *et al.* Biliary sclerosis in patients receiving hepatic arterial infusions of floxuridine. *J Clin Oncol* 1985, **3**, 98–102.
17. De Takats PG, Kerr DJ, Poole CJ, Warren HW, McArdle CS. Hepatic arterial chemotherapy for metastatic colorectal cancer. *Br J Cancer* 1994, **69**, 372–378.
18. Marinelli A, van de Velde CJH, Kuppen PJK, Franken HCM, Souverijn JHM, Eggermont AMM. A comparative study of isolated liver perfusion versus hepatic artery infusion with mitomycin C in rats. *Br J Cancer* 1990, **62**, 891–896.
19. De Brauw LM, Marinelli A, van de Velde CJH, Hermans J, Tjaden UR, Erkelens C, De Bruijn EA. Pharmacological evaluation of experimental isolated liver perfusion and hepatic artery infusion with 5-fluorouracil. *Cancer Res* 1991, **51**, 1674–1700.
20. Sindelar W. Isolated-perfusion of the liver with 5-fluorouracil. *Ann Surg* 1985, **201**, 337–343.
21. Marinelli A. Isolated liver perfusion treatment of hepatic metastases. Experimental and clinical studies. Thesis, University of Leiden, The Netherlands, 1992.
22. Aigner KR, Walther H, Link KH. Isolated liver perfusion with MMC/5FU—Surgical techniques, pharmacokinetics, clinical results. *Contr Oncol* 1988, **29**, 229–246.
23. Spears CP, Gustavsson BG, Berne M, Frösing R, Bernstein L, Hayes AA. Mechanisms of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. *Cancer Res* 1988, **48**, 5894–5900.
24. Swain SM, Lippmann ME, Egan EF, Steinberg SM, Allegra CJ. Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J Clin Oncol* 1989, **7**, 890–899.
25. Johnston PG, Fisher E, Rockette HE, *et al.* The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* 1994, **12**, 2640–2647.
26. Lenz HJ, Leichman C, Danenberg P, Silberman H, Horikoshi T, Kiyabu M, Danenberg K, Spears CP, Laine L, Fuerst M, Leichman L. Thymidylate synthase (TS) gene expression predicts response of primary gastric cancer (GC) to 5-fluorouracil (5FU)-leucovorin (LV)-cisplatin (DDP). *Proc Am Soc Clin Oncol* 1993, **12**, 199.
27. Tominaga T, Toi M, Shirasaka T. Enhanced inhibition of thymidylate synthase by 5-fluorouracil and [6S]leucovorin combination therapy for breast cancer. *Anticancer Res* 1994, **13**, 2425–2428.
28. Peters GJ, van der Wilt CL, Van Groeningen CJ, Smid K, Meyer S, Pinedo HM. Thymidylate synthase inhibition after administration of 5-fluorouracil with or without leucovorin in colon cancer patients:

implications for treatment with 5-fluorouracil. *J Clin Oncol* 1994, **12**, 2035–2042.
29. Marquet R, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon carcinoma. *Int J Cancer* 1984, **38**, 689–692.
30. Marinelli A, Pons DHA, Kuppen PJK, Vreeken JAC, Tjaden UR, van de Velde CJH. Isolated liver perfusion (ILP) vs hepatic artery infusion (HAI) with 5-Fluorouracil (5FU) and mitomycin C (MMC) in a rat model. *Proc Am Ass Cancer Res* 1990, **31**, 429.
31. 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.
32. Spears CP, Shahinian AH, Moran RC, Heidelberger C, Corbett TH. *In vivo* kinetics of thymidylate synthetase inhibition in FUra-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res* 1982, **42**, 450–456.
33. van der Wilt CL, Pinedo HM, Smid K, Peters GJ. Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumours. *Cancer Res* 1992, **52**, 4922–4928.
34. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989, **24**, 148–154.
35. Berne MHO, Gustavsson BG, Almersjö O, Spears CP, Frösing R. Sequential methotrexate/5FU: FdUMP formation and TS inhibition in a transplantable rodent colon adenocarcinoma. *Cancer Chemother Pharmacol* 1986, **16**, 237–242.
36. Chu E, Koeller DM, Johnston PG, Zinn S, Allegra CJ. Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-γ. *Mol Pharmacol* 1993, **43**, 527–533.
37. Chu E, Koeller DM, Casey JL, Drake JC, Chabner BA, Elwood PC, *et al.* Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. *Proc Natl Acad Sci USA* 1991, **88**, 8977–8981.
38. Weber G. Biochemical strategy of cancer cells and the design of chemotherapy: G.H.A. Clowes memorial lecture. *Cancer Res* 1983, **43**, 3466–3492.
39. Johnston PG, Drake JC, Steinberg SM, Allegra CJ. Quantitation of thymidylate synthase in human tumors using an ultrasensitive enzyme-linked immunoassay. *Biochem Pharmacol* 1993, **45**, 2483–2486.
40. Aherne GW, Hardcastle A, Newton R. Measurement of human thymidylate synthase (hTS) in cell lines using ELISA. *Ann Oncol* 1992, **3** (Suppl. 1), 77.
41. Horikoshi T, Danenberg KD, Stadlbauer THW, Volkenandt M, Shea LCC, Aigner K, *et al.* Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. *Cancer Res* 1992, **52**, 108–116.
42. Aigner KR. Isolated liver perfusion: 5-year results. *Reg Cancer Treat* 1988, **1**, 11–20.
43. Marquet RL, Jeekel J. Combined effect of 5-fluorouracil and interferon on experimental liver metastases of rat colon carcinoma. *J Cancer Res Clin Oncol* 1985, **109**, 156–158.
44. Busch ORC, Slooter GD, Jeekel J, Marquet RL. Effect of levamisole and 5-fluorouracil in immune status and tumor growth in a rat model of colon carcinoma (CC531) in the rat. *Proc Am Ass Cancer Res* 1993, **34**, 457.
45. Houghton JA, Williams LG, Loftin SK, Cheshire PJ, Morton CL, Houghton PJ, Dayan A, Jolivet J. Factors that influence the therapeutic activity of 5-fluorouracil [6RS] leucovorin combinations in colon adenocarcinoma xenografts. *Cancer Chemother Pharmacol* 1992, **30**, 423–432.

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Coenzymes Q_9 and Q_{10} in Skeletal and Cardiac Muscle in Tumour-bearing Exercising rats

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Physical exercise increases metabolic rate, and induces both adaptational biogenesis of mitochondria in skeletal muscle and an increase in antioxidant capacity. The onset of experimental anorexia and cachexia can be delayed by voluntary exercise. As skeletal muscle is the main target for cancer cachexia, we determined the levels of coenzymes Q_9 and Q_{10} in skeletal muscle from tumour-bearing exercising rats, and compared them to those of sedentary tumour-bearers and controls. Both tumour-bearing groups had increased levels of coenzymes Q_9 and Q_{10} in the anterior tibial muscle ($P < 0.05$ for exercised animals). In the soleus muscle, only the tumour-bearing exercising animals demonstrated an increase in the levels of both coenzymes ($P < 0.05$). In cardiac muscle, the presence of tumour and exercise reduced the levels of coenzymes below that of sedentary controls. Exercise counteracted the anaemia in the tumour-bearing host ($P < 0.05$). In conclusion, the increase in antioxidant capacity in skeletal muscle indicates a defence mechanism in the tumour-bearing hosts which is augmented by physical exercise.

Key words: exercise, cancer, ubiquinone, energy metabolism
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